

## Maintaining a global vision

**The rules on sharing large-scale facilities are under pressure. Neither politicians nor scientists should ignore the role of self-interest. But respect must, where possible, still be maintained for universal values.**

There is a troublesome conflict of principle bubbling beneath what might otherwise seem to be an innocuous issue: the rules of access to large and, increasingly, medium-sized, publicly funded research facilities. At stake is the question of whether access should be determined primarily by scientific merit, or by the interests of those who have sponsored the facility. In an ideal world, the two would be congruent. In reality, the pressures of self-interest that such rules are increasingly written to reflect can, without proper safeguards, end up threatening those universal values on which the health of science depends.

The lesson is brought home sharply by a recent report prepared for the Megascience Forum of the Paris-based Organization for Economic Cooperation and Development (OECD). The report was commissioned at a time when certain constituencies in the United States were balking at the idea of having to contribute to the construction of the Large Hadron Collider at CERN, the European Laboratory for Particle Physics, in Geneva. The argument being used was that, since foreign researchers were able to compete for the use of many major US science facilities without having to pay for their construction, European scientists should conduct the same 'open door' policy in return.

The OECD report concluded, after studying a wide range of facilities, that there is no universally appropriate set of access rules, implicitly rejecting the arguments that everyone should adopt the US principle that access be based purely on scientific merit. It is a timely reminder that the more utopian ideals of a single, global scientific community are frequently at variance with the growing demands to show value for money in the use of public funds.

The key question, of course, is: value to whom? It is difficult to convince a small country that pays a relatively large proportion of its science budget as a subscription to an international facility — such as CERN — that its interests are being met through experiments conducted by scientists from a large, non-paying state. This is particular-

ly so if its own scientists are, for whatever reason, badly placed to conduct comparable experiments themselves.

It is not only international facilities that experience tension. In the United States, synchrotron facilities have traditionally been built by the Department of Energy. This reflects both the disciplinary skills required for the machines' construction and the fact that much of their use has been in the physical sciences. Their increasing use by biologists has inevitably raised questions about the fairness of the traditional distribution of responsibility, particularly at a time of burgeoning political support for the biomedical sciences. It is thus entirely appropriate that the National Institutes of Health should make a contribution to future construction costs (see page 203).

Such issues are likely to grow, partly reflecting the increased interest in biology in recent decades. Particle physics and space exploration have been joined as big spenders by areas such as human genome sequencing and biodiversity research that, while still of global interest, cannot easily be categorized as 'big science'. The instrumentation needs of 'small science' are also growing, to the extent, for example, of leading to demands for regional nuclear magnetic resonance spectrometers in the United States (see page 201).

The merits of funding such facilities, where needed, on a regional basis are obvious. And, from a purely national perspective, the purview of a single funding agency would, hopefully, ensure that access was not determined solely by ability to pay. At the international level, however, the global perspective provided by the value of large scientific projects in helping to forge political alliances has largely disappeared in a world no longer dominated by competition between power blocs. As the terms of self-interest move from the political to the economic, it is important to find new ways of supporting the universal ideals from which science has taken so much strength — for example by allowing facility managers a degree of freedom in allocating research time to the 'worthy but needy'. □

## Pie in the sky

**Ageing astronauts should be more honest about their motivations for wishing to return to space.**

Few people actually believe that Senator John Glenn's recent space shuttle jaunt was conducted primarily in the interests of science. But since he and the US space agency NASA repeatedly and adamantly defended his flight on those grounds, it deserves comment from the research community. For while NASA and the Senator apparently believe that any attention to science is positive, their overhyped claims mean that public understanding of what makes a well designed experiment may well have taken a step backward.

The flight undoubtedly succeeded in calling attention to parallels between the physiological effects of ageing and the 'deconditioning' that occurs in weightless astronauts. That line of inquiry may even be promising if humans continue to travel into space. But calling the experiments in which Glenn participated 'cutting edge' research on ageing misrepresents how scientific studies are conducted.

None of the shuttle experiments required a geriatric test subject. Before Glenn lobbied his way onto the flight, NASA had no plans to

send an older astronaut into space. In fact, the agency had pushed at least one shuttle veteran in his sixties — and with far more spaceflight experience than Glenn — out of the door. Furthermore, flying one old person in space doesn't yield statistically valid conclusions about old people in general. And NASA has no plans for follow-up studies with other test subjects of a similar age.

Glenn is surely aware of the limited scientific value of his flight. But he desperately wanted to return to orbit, and seized on science as a convenient way to make his joyride sound more high-minded than it really was. Ironically, he didn't need the fig-leaf. The American public seemed more than willing to give an ageing hero his 'victory lap' without any trumped-up justification. Why harp on the research, then? The astronaut-turned-Senator-turned-astronaut has said his next goal will be to help combat what he views as apathy and cynicism among young people. Perhaps a good starting point would be for public figures to be more honest about their motivations. □



# US researchers push network of massive spectrometers

[WASHINGTON] An ambitious plan for ten regional 'collaboratories', each equipped with powerful nuclear magnetic resonance (NMR) spectrometers, has been proposed by an informal group of US scientists. The move is based on the belief that high-field NMR is set to emerge as a critical research tool for structural biologists, geneticists and materials scientists.

The group, led by the National High Magnetic Field Laboratory at Florida State University at Tallahassee, suggests that the US government should spend \$110 million on establishing the new centres and equipping them with spectrometers that can measure proton resonance at 900 Mhz.

A further \$150 million would be spent later to provide even better spectrometers that could operate at 1 GHz, a higher performance than is currently obtained by any actual or planned NMR machine.

NMR spectrometers, which can determine the structure of molecules that cannot be synthesized as single crystals for observation by the more established means of X-ray crystallography, improve in resolution as the cube of their operating frequency. Incremental increases in frequency therefore greatly increase their usefulness, advocates of the approach point out.

But the powerful superconducting magnets that drive NMR spectrometers are difficult to build because of the immense stresses to which they are subjected as the frequency of the machines is raised.

An order for what would be the most powerful NMR spectrometer in the world — a 900-MHz machine to be installed at the Department of Energy's Pacific Northwest National Laboratory (PNNL) in Washington



A matter of size: larger machines may be needed than those available in individual labs (above).

State — is running two years late.

US sources say the contractor, UK-based Oxford Instruments, has lost money on the \$7 million order, and replaced the head of its NMR unit earlier this year as a result of problems in delivering the order. The new head of the unit, Alan Street, says the magnet will undergo a new round of testing this week, and that it should be delivered next summer.

These technical problems, together with what agency officials describe as a narrow base of support for NMR spectroscopy among the scientific disciplines to which it should be of most value, means that the proposal for ten regional centres has been met with scepticism in Washington.

But Jack Crow, director of the Tallahassee laboratory, says the time is right for the government to plan a series of national centres to

meet future scientific needs. He compares the current status of NMR to the earlier evolution of X-ray spectroscopy, when scientists switched from inexpensive equipment at their own university departments to the far more powerful synchrotrons at central government-run facilities.

Many university departments have NMR spectrometers that operate at 300 or 400 MHz, says Crow, and some can afford 750-Mhz machines. Spectrometers of 900 MHz or more will be too expensive and complex for single universities to operate, he says, but scientists will still want access to them.

According to the report, the larger machines will allow biologists to solve the structures of proteins with molecular weight of 100,000 dalton, twice today's upper limit. They would also generate new structures more rapidly, it says.

Crow and 20 other NMR specialists produced a report outlining their proposal in August, at the suggestion of the National Science Foundation (NSF). The report says that the 'collaboratorium' — a US term describing centres whose participants communicate over high-speed Internet links — would be the best way to distribute access to the spectrometers, which would themselves be distributed across the United States.

The report also argues that more powerful NMR machines will benefit materials scientists, and would enable biologists to solve the problems of structure needed to improve understanding of gene function and regulation, as well as issues in neuroscience. But agency officials remain to be convinced that an expensive network of state-of-the-art facilities is justified at this stage.

The "case has not yet been made" for the network, says Robert Eisenstein, head of the mathematical and physical sciences directorate at the NSF. NMR facilities that can operate at 1 GHz "will be needed at some point, but not today", says Mary Clutter, head of biological sciences at the foundation.

Others are more upbeat. Marvin Cassman, director of the National Institute of General Medical Sciences at the National Institutes of Health, says the value of NMR spectroscopy will increase as the available field increases, and that he supports further discussion of the proposal.

Ari Patrinos, head of biological and environmental sciences at the Department of Energy, says of the high-field NMR spectrometers: "I don't think they'll have as prominent a role as light sources, but they are an element of the panoply of tools that we'll use".

Colin Macilwain

## Climate conference agrees action plan

[LONDON] The fourth conference of the parties to the United Nations climate convention ended in Buenos Aires last week, with agreement on a plan of action and a deadline of the year 2000 to finalize mechanisms for implementing the Kyoto protocol.

These include mechanisms for trading of greenhouse-gas emissions between developed countries, help for developing countries with clean energy projects, funding on technology transfer, and help from the global environment facility for countries adapting to climate change.

The United States, which last week became the sixtieth country to sign the

Kyoto protocol, made some progress in its attempts to make developing countries undertake voluntary commitments to reduce greenhouse-gas emissions.

The Group of 77 countries prevented this issue from becoming a formal agenda item. But it failed to prevent the host country, Argentina, followed by Kazakhstan, from breaking ranks and agreeing voluntary commitments to reduce emissions.

The protocol will not become legally binding until 55 per cent of developed and developing countries sign the protocol and ratify it in their national parliaments. So far, this has been done in just two countries: Fiji, and Antigua and Barbuda.

Ehsan Masood

# Interdisciplinary research 'being stifled'

[STOCKHOLM] The traditional disciplinary organization of research bodies is not only failing to encourage interdisciplinary research, but is stifling it, leading scientists and research administrators were told last week at a symposium in Stockholm.

Yet this is happening at a time when a growing need for more holistic approaches to both fundamental scientific questions and complex socioeconomic issues — such as public health, global warming and biodiversity — is making interdisciplinary research more necessary than ever.

Those who attended the meeting, organized by the Wenner-Gren Foundation and the National Science and Engineering Research Council of Canada, included Rita Colwell, director of the US National Science Foundation (NSF) and Sydney Brenner, the former director of the UK Medical Research Council's Laboratory of Molecular Biology in Cambridge. Colwell described interdisciplinary research as "nothing short of vital" and identified it as "one of the major challenges for NSF in the coming years".

Many excellent interdisciplinary research centres and projects have been established over the past few decades. But the main message of the meeting was that these represent an 'avant-garde' minority, while the bulk of mainstream research remains organized along disciplinary lines.

A succession of speakers told the meeting

that the current system tends to discriminate against researchers who dare to move beyond the strict confines of their narrow corner of intellectualism. "The departmental structure is a block," said Colwell. "We need to tear down the walls that separate our funding agencies."

The meeting heard that the need to remove these barriers is urgent, as research needs are undergoing a 'paradigm shift' towards the understanding of complex systems. A major need identified in the post-genome era, for example, is for an effort to reintegrate advances in genetics and molecular biology with those in other areas to get a better picture of how whole organisms work.

A more sophisticated multidisciplinary approach to health and education, integrating neurobiology, developmental biology and epidemiology, was also identified as a major need by several speakers, including Lennart Philipson, from the Karolinska Institute in Stockholm, and Fraser Mustard, head of the Canadian Institute for Advanced Research in Toronto.

Mustard argued that, while most biomedical research uses a disease-based model, major opportunities exist in a broader approach that seeks to explain a growing body of evidence that most of the variation in health and mortality in adulthood is not linked to conventional medical risk factors, but to the environment during early life.

Pioneering interdisciplinary work in this area, by Michael Marmot's group at University College London, has been widely praised. But it is experiencing difficulties in attracting grants, said Mustard. "This research could open up a whole new area of medicine, but it would contradict the dominant disease-based approach."

One problem identified was that interdisciplinary applications for grants are usually assessed by a series of discipline-based peer review committees rather than by interdisciplinary panels, with the result that good projects fall between the cracks. The disciplinary nature of most primary journals means that interdisciplinary researchers find it difficult to have work published in ranked journals.

The meeting produced few remedies to encourage more interdisciplinary research. But there was wide suspicion of the quality and cost-effectiveness of top-down interdisciplinary initiatives by research institutions. "Efforts to create marriages in heaven too often end up with labs sharing the same address, but not a life," said Jeremy McNeil, a biologist at Laval University in Canada.

"Top-down initiatives often lack the motivation of someone trying to address a specific problem and who doesn't care what discipline you use," said Brenner. His experience had convinced him that what was important was to hire the best staff, and ensure "that young people can learn from other specialists".

But McNeil pointed out that larger programmes on global issues, such as biodiversity and global warming, involved extensive cross-agency and university support, and therefore required a top-down approach. Indeed, the meeting identified the major challenge ahead as managing global interdisciplinary research projects on important socioeconomic issues.

Lessons learned from a study of Canadian fisheries management programmes included the imperative need to involve social scientists at every stage, and to avoid compartmentalization of the scientific assessment, political and management phases.

Past fisheries management has been a "total disaster," said Hugh Morris, from the Padre Resources Corp. in Canada, because it failed to take into account the full complexity of the marine ecosystem. "Wider democratization might give you better science," he added, arguing that the inclusion of social scientists in recent programmes has helped to question the assumptions scientists make.

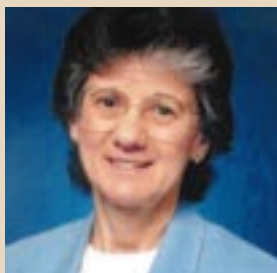
Finding a common language — or "scientific Esperanto" between disciplines, as Colwell put it — to avoid the worsening jargon was identified as a major challenge. "We cannot afford to speak in the tongues of our own disciplines," said Colwell. **Declan Butler**

## NSF boss champions need for teamworking

[STOCKHOLM] Interdisciplinary researchers have found a strong ally in Rita Colwell, the new director of the US National Science Foundation. Colwell flew to last week's meeting (see above) from Bangladesh, where, on a grant from the National Institutes of Health, she has been crowning the success of an interdisciplinary programme on cholera that she began 25 years ago.

Colwell told the meeting that she had had major difficulties over 20 years getting funding. But, now that the success of her project was accepted, "I've gone from being a pariah to being a guru."

Colwell observed that cholera epidemics always spread inland from the coast. Her hypothesis, that the bacterium *Vibrio cholerae*



Colwell: from 'pariah to guru'.

might be associated with plankton blooms, was at first scorned. But it is now the basis of a global project involving space scientists, oceanographers, molecular biologists, social scientists, physicians and high-speed computing scientists.

Colwell showed that *Vibrio cholerae* was associated with gravid copepods where it played a role in breaking open the egg sac by secreting

chitinases. She showed that cholera epidemics were correlated with the seasonal incidence of the bacteria in the copepods.

Colwell's group has since used this insight to develop a global predictive system for cholera epidemics using remote sensing techniques. In Bangladesh last week, she was working on prevention. She tested local fabrics as potential filters and found that sari fabric, folded ten times, can remove over 90 per cent of cholera bacteria.

Colwell is now working with social scientists to implement an education programme to introduce the filters. The lesson, she says, "is the need to appreciate the complex reactions that characterize ecosystems; too complex for any one discipline". **D.B.**



## NIH promises funds to help biologists to use beamlines

[WASHINGTON] The US National Institutes of Health (NIH) is set to provide a major infusion of equipment and technical staff to help biologists working at five synchrotron light sources operated by the Department of Energy (DOE).

Harold Varmus, director of the NIH, told a meeting last week of health research lobbyists: "We'll be making a substantial contribution" to the synchrotron facilities "in the future, and perhaps this year".

The NIH contribution may be as much as \$20 million in the current financial year, which ends in September 1999. It is expected to help equip beamlines at the synchrotrons, to upgrade some of the facilities, and to pay for support staff to help structural biologists use them.

It has been estimated that a capital investment of \$12 million and an extra \$5.5 million in annual operating costs would double the amount of time available on the beamline for structural biologists at the synchrotrons (see *Nature* 393, 3; 1998).

The increased NIH contribution confirms that the biomedical research agency, which last month received a mammoth \$2 billion funding increase from the Congress, is ready to help the cash-strapped DOE with facility operations.

The DOE built most of the 'big science' instruments in the United States, including the synchrotrons, chiefly for use by physicists and materials scientists. But an increasing proportion of their users are NIH-supported biologists.

A panel of officials established in the spring to look into the question of inter-agency cooperation on synchrotron operations recently sent its report to the Office of Science and Technology Policy at the White House. The agencies involved are now trying to implement its findings during the current financial year.

Marvin Cassman, director of the National Institute of General Medical Sciences at NIH, says: "I do anticipate we'll be making a substantial contribution". But he adds that its precise value remains "a little unclear" and that he is "still not certain we can do it this year". NIH is currently scrambling to allocate its unexpectedly large budget increase.

The facilities that will benefit from the news include the Advanced Photon Source at the Argonne National Laboratory, Illinois; the Advanced Light Source at the Lawrence Berkeley laboratory in California; the Stanford Synchrotron Radiation Laboratory at Stanford, also in California; and the National Science Foundation's synchrotron at Cornell University, New York State. **Colin Macilwain**



Deep blue: light is emitted from the core of the ILL reactor as a result of Cerenkov radiation.

## European reactor accepts US demands on fuel shift

[MUNICH] The Institut Laue Langevin (ILL) in Grenoble, France, has bowed to US pressure and agreed to convert its high-flux 57 MW research reactor to use low enriched uranium (LEU) rather than 'bomb-grade' highly enriched uranium (HEU). The move follows the institute's failure to secure sources of HEU from Russia.

According to a memorandum of understanding signed on 12 November, the conversion will take place "when it becomes technically and economically possible". In return, the United States will supply HEU to the reactor until it converts, and also take back spent fuel.

The Reduced Enrichment for Research and Test Reactors (RERTR) programme, which started in 1978, requires the United States to promote the development of LEU fuel for reactors wishing to convert. The programme is intended to reduce international commerce in bomb-grade uranium.

It was strengthened in 1992 by the so-called Schumer Amendment, banning the delivery of US HEU to reactors that refuse to cooperate with the RERTR programme. The amendment was named after Democrat Congressman Charles Schumer, who earlier this month defeated incumbent Alfonse D'Amato to win a seat in the US Senate, representing New York.

As a result of US pressure, most research reactors have converted to existing LEU fuel or have agreed to work with US scientists to develop LEU fuel which they can use without loss of performance or extra cost. The ILL reactor, which provides neutron beams for structural analysis for scientists from ILL's seven member states, was one of the few to hold out, partly because France objected to US interference in European affairs.

When the ILL restarted operation in 1995 after a four-year shutdown for repair, it found its normal fuel supply blocked by the Schumer agreement. ILL officials turned to Russia for HEU supplies rather than accept US terms, and Russia became an associate member of ILL in November 1996 in exchange for supplying HEU from Minatom, the Russian Atomic Agency.

But the Russian fuel failed to materialize and ILL suspended Russian membership at the beginning of this year. Dirk Dubbers, the director of the ILL, cites "problems between the Russian science ministry, which benefited from the scientific opportunities, and Minatom, which saw no exchange of cash".

The agreement between ILL and the United States took observers by surprise. A spokesman for Greenpeace International described it as "welcome, even if not motivated by non-proliferation concerns".

The European Union's Petten research reactor in Belgium and Belgium's national research reactor BR2 have long expressed interest in conversion, but have not yet agreed to do so. If they do convert, the only remaining European reactor to hold out will be the controversial German research reactor FRM II, being built by the Technical University of Munich.

The operators of FRM II have cited the reluctance of ILL to comply with US terms for HEU supply as support for their reluctance to do so. But the ILL's change of heart comes on top of a statement from the new red-green federal government in Germany saying that use of bomb-grade uranium in research reactors is "problematic and dubious in terms of foreign policy". The statement says the government will check again whether FRM II could be converted to LEU. **Alison Abbott**

# Berlin university shakes off radical past...

[BERLIN] The Free University in Berlin, which celebrates its fiftieth birthday next month, is engaged in major reforms to eliminate the excesses in teaching and research that have been a legacy of its turbulent history.

The university was established in 1948 in the British sector of occupied Berlin when students in the west of the city were denied access to the University of Berlin (now Humboldt University) in the Russian sector.

Generous subsidies from the federal government meant the university grew rapidly. Its isolation from the rest of West Germany encouraged an independent spirit to flourish, and this was responsible for many radical changes following the student protest movements of 1968.

With a post-reunification decision to halve its size, however, those running the university are taking the opportunity to reverse many of these changes and introduce new rules to improve its research and teaching performance.

"The Free University was a playground for experiments in education and science policy, and all the errors made [in Germany] are nowhere more apparent," says Peter Gaetgens, the university's vice-president.

Under pressure from students in the late 1960s, the number of exams was reduced, while low attendance at lectures lengthened the average study time to eight years. Inward-looking recruitment practices weakened research, assistant professors being promoted to full professors with few of the normal rules governing academic selection.

As a result, the number of faculty mem-

bers soared, while the quality of research was diluted. "In the rush of hiring, too many professors came on board who were never productive in research," says Gaetgens.

Not all departments suffered equally. Physics avoided the excesses of the period, maintaining a strong research profile and recruiting good professors from outside.

Biology suffered more. This large department, with 46 chairs, became mired in internal disputes. The 'democratization' of faculty committees left professors in a minority, while conflicts between political ideologies distracted academics from research.

Neuroscientist Randolph Menzel, one of the few biologists hired from outside Berlin during the 1970s, says "the Free University got so much money thrown at it through the 1970s and 1980s that it never developed a culture of competition". Even now, he says, "some of the biology faculty won't accept publication record as an indication of research success".

But with the country's reunification in 1990, the flow of money stopped, and the Free University found itself with few friends in high places. It fought to maintain its operational style with an unsympathetic Berlin Ministry for Science and Research (see *Nature* **370**, 167; 1994). But in the last couple of years the university has accepted the new reality and begun serious and deep reforms.

Teaching is being improved and regularly evaluated, and students are given more frequent exams and are encouraged to complete their studies in four years.

Steps have also been taken to improve the

quality of research, with reorganization made possible by a change in university rules putting more decision-making power in the hands of deans and rectors, at the expense of faculty committees. Already time spent in committee meetings is reduced. According to a commonly quoted calculation, this used to cost the university DM3 million (US\$1.8 million) per year in academics' time.

To adapt to its smaller size, the Free University has redesigned its internal structure, with fewer, broader, faculties. Chemistry, biology and pharmacy have been grouped in a new life sciences faculty to encourage cross-fertilization of ideas. But not everyone is happy. Inorganic chemists worry that they will be marginalized in a life sciences faculty.

Most departments have started to allocate their own research funds in line with performance, based on the amount of external grants already won: unproductive professors get nothing.

The university still has many relatively unproductive professors. A ranking of universities by grant money won between 1991 and 1995 places it fifth among 68 universities funded by the Deutsche Forschungsgemeinschaft (DFG), Germany's university research grant agency. When recalculated on a per professor basis, it falls to number 36. On this basis, it is eighteenth out of 59 in the natural sciences, and thirty-fourth out of 51 in biology and medicine.

On the other hand, the university receives an above average number (nine) of the DFG's coveted 12–15-year grants (*Sonderforschungsbereiche*), and nine researchers at the Free University have won the DFG's prestigious Leibniz awards, showing that pockets of research excellence exist, without which the university's ranking would be yet lower.

The university is trying to build on this excellence and clear its dead wood by waiting for the large number of professors hired in the 1970s to retire, and replacing them with active and successful researchers. But for the large cut in faculty numbers — by 2003 the number of chairs funded by the Berlin government will fall from 750 to 360 — this would be easy. The 1970s professors are approaching retirement *en masse*, and Berlin has become an attractive place for scientists to live and work.

The university's community of active researchers say that the large-scale cuts forced on the Free University by circumstance have been healthy rather than damaging because of the urgently needed reforms they precipitated.

"At first we thought that the big tanker that is the Free University could not be turned," says Günther Kaindl, a professor of solid state physics. "But it seems that it was mobile after all."

**Alison Abbott**

## ... and hopes for the gift of a new home



[BERLIN] The Free University is hoping for a fiftieth birthday present — the nearby barracks (above) which was the US Army headquarters until the end of the Cold War.

Founded at the start of the Cold War, the university grew haphazardly among villas vacated after World

War II in the Berlin suburb of Dahlem. History dictated its lack of a real centre.

"It would be a nice symbol of our future if the new government gave us the buildings", says Peter Gaetgens. "The years 1948, 1968 and 1990 were important cornerstones of

our history, but they are the past."

The government of Berlin supports the idea. But the federal government is as yet undecided. It is toying with the idea of moving the German secret service into the buildings instead.

**A. A.**





Going for growth: Japan's prime minister Keizo Obuchi unveils his new spending plans.

## Economic package will boost R&D infrastructure

[TOKYO] Science and technology are among the beneficiaries of the Japanese government's largest-ever economic stimulus package, which was unveiled on Monday (16 November) and aims at kick-starting the nation's ailing economy.

The package aims to create one million jobs and restore economic growth by the end of the 1999 fiscal year. The core of the measures focuses on public works projects and rescuing the ailing banking and business sector. But the government's initiative includes provisions for creating businesses through 'commercially applicable research'.

The public works projects will include the construction of new campuses at national universities and plans to build an 'international science village', a project supported by science-related ministries to create an international centre to promote scientific research (see *Nature* 393, 5; 1998).

The ¥24 trillion (US\$204 billion) package features ¥8.1 trillion for a 'social infrastructure programme' aimed at developing the infrastructure needed for areas such as telecommunications, science and technology, education and the environment.

The programme includes plans to construct information highways — a project to create fibre optic networks — and a 'next-generation transport' system, as well as plans to support 'future technology', especially biotechnology.

A significant portion of the funds will go towards projects linked to environmental protection, including research into the effects of endocrine disrupters which the government has listed as one of its top priorities (see *Nature* 392, 748; 1998).

Keizo Obuchi, Japan's prime minister, said the package is "the first step towards directing the Japanese economy back on a path of sustainable economic growth within two years". The measures are expected to be financed by the government's third supplementary budget, details of which are expected to be announced this month. **A.S.**

# Private deal puts Japanese researcher in hot water

[TOKYO] Allegations of corruption involving a former professor from one of Japan's leading universities and executives of a major drug company are casting a long shadow over the government's efforts to promote collaboration between industry and the academic community.

The allegations have also thrown a spotlight on the extent to which activities considered as normal practice elsewhere are still regarded with suspicion in Japan.

Akihiko Otsuka, the president of Otsuka Pharmaceutical Co., one of Japan's largest drug companies, was arrested last week over payments made to Hiroyoshi Hidaka, a former professor at Nagoya University Medical School. Hidaka is said to have received large sums for providing 'technical consultation' for the development of drugs.

Hidaka, one of Japan's leading pharmacologists, had already been indicted in August for accepting money from two other drug companies — Fuji Yakuhin Co. and Nippon Shinyaku Co. — from which he received ¥124 million and ¥60 million (US\$1 million and US\$490,000) respectively. The two companies, like Otsuka, claim that the payments were 'technical guidance fees' related to drug research and development.

The arrests of Otsuka and two senior executives from the company's drugs development division have shocked Japan's pharmaceutical industry, and fuelled media criticism over suspected corruption and cronyism between industry and universities.

According to Nagoya District Prosecutor's Office and the prefectural police, the three are suspected of paying Hidaka ¥72 million between 1995 and 1998 through a 'dummy' medical research company run by an acquaintance of the former professor.

Hidaka, who resigned from Nagoya University four days before his arrest in August, faced a fresh warrant last week for allegedly taking the money from Otsuka. The company is said to have approached Hidaka in 1993 to carry out collaborative research to develop a drug to replace its best-selling anti-platelet therapy — which Hidaka had helped to develop — before its patent expired in 1999.

According to allegations in the investigators' report, Hidaka conducted experiments within the university and provided the company with data on the development of the new drug, as well as allowing researchers from Otsuka to carry out research using facilities at the university laboratory.

Such activities are forbidden under Japanese law, which prohibits employees of national universities from taking part in profit-making activities. Also prohibited are

exploitation of university facilities, equipment and/or staff for commercial purposes, and any involvement of industry researchers in activities other than private research.

Investigators are describing the money paid to Hidaka as "bribes" on the basis that he accepted payments in return for allowing companies to exploit university facilities for commercially-orientated research, and because the dummy medical company to which the companies paid the fees did not provide technological training as it claimed.

Otsuka and his two employees have acknowledged paying the money, but deny that it constituted a bribe. "The payments were based on a formally signed contract and memorandum to obtain technological guidance (from Hidaka), and were never meant to be bribes," said a managing director of Otsuka last week.

But he later added: "While we did not consider any of the agreement we made as being corrupt, most of us were aware that it could easily trigger misleading ideas."

The Ministry of Health and Welfare has expressed concern over the incident, saying that the suspected corruption could affect the nation's trust in the safety and reliability of pharmaceutical products.

"It is regrettable that such cronyism existed between pharmaceutical companies and a basic researcher. It is important that industry and researchers act morally and stick to the rules when they carry out collaborative research," says a health ministry spokesman.

Hidaka received a warning from the Ministry of Education, Science, Sports and Culture (Monbusho) in 1985 for carrying out similar activities involving collaborative research with pharmaceutical companies.

"It is ironic that Hidaka and the company executives have been arrested at a time when the government is placing so much effort in promoting university/industry collaboration," says one professor from Osaka University Medical School. "What they are alleged to have done was not necessarily uncommon; it seems that they were at the wrong place at the wrong time."

Since a law promoting collaboration between universities and industry was passed in May, Monbusho and the Ministry of International Trade and Industry have worked towards relaxing the civil service law to encourage ties between university researchers and industry (see *Nature* 390, 105; 1997).

But scientists say the status of researchers at national universities needs to be clarified. Otherwise they will not be able to differentiate the thin division between 'collaboration' and 'corruption'. **Asako Saegusa**

# Cannabis laws 'threaten validity of trials'

[LONDON] Clinical trials into the medical effects of cannabis could be in jeopardy unless current regulations are either changed or interpreted more flexibly, according to British scientists planning the trials.

The outcome of the trials will help determine whether the government bows to calls to let doctors prescribe cannabis. The government last week rejected the latest such call, in a report from the House of Lords, saying that such a move depends on whether the medical benefits of cannabis are confirmed by scientific research.

The trials are planned under the aegis of the Royal Pharmaceutical Society in response to widespread — but primarily anecdotal — reports that cannabis is effective in pain relief, and that it helps to relieve the symptoms of multiple sclerosis (MS) sufferers.

Separate trials will attempt to establish and quantify these benefits. Protocols for the trials are being worked out by a group set up by the society. But whether the trials take place depends largely on the interpretation of requirements attached to awarding licences for cannabis research.

Applicants for a licence need to convince the Home Office that the drug will not be sold or used for any purpose other than the experiment. A further requirement is that the cannabis be administered, preferably in medicinal form, in the presence of a doctor or pharmacist in a specified location, usually a hospital or laboratory.

One consultant neurologist in London says the first condition is virtually impossible to meet, as most patients are already using the drug to alleviate their symptoms.



Roger Pertwee, reader in biomedical sciences at the University of Aberdeen, a former president of the International Cannabinoid Research Society and a member of the trials working group, highlights another difficulty. He says it is "essential" that patients on the MS trial can administer the drug at home, which current regulations do not allow.

Similarly, smoking the drug is known to be far more medically effective than the best available alternative of a tablet made by isolating the hydrocarbon cannabinoid  $\Delta^9$ -THC, the main psychoactive compound of cannabis. But members of the working group acknowledge that the Home Office is unlikely to sanction large-scale trials involving cannabis being smoked.

Government officials acknowledge that the issue will not be easy to deal with under the current regulations. But they are confident that the trials will go ahead as planned,

either in an outpatient arrangement or even at patients' homes.

"There are no statutory conditions to research licences other than the need for safe custody [of the drug], a finite time for the experiment, which should be conducted from a hospital, approval of the experiment by an ethics committee, and the keeping of records," says one official.

Last week's report from the House of Lords Select Committee on Science and Technology concludes that the legalization of medicinal cannabis will give a further boost to research. The report says that such a move should help remove the stigma felt by researchers of working with an illegal drug.

It adds that the legalization of medicinal cannabis will make it easier to get a research licence to study cannabis, and boost clinical trials by enabling cannabis users to come forward to take part. But professional bodies representing research scientists and pharmacists have reacted more cautiously.

The Royal Society says cannabis should remain banned until there is sufficient evidence of its medical benefits, irrespective of the impact on research.

The Medical Research Council and the Royal Pharmaceutical Society take a similar view, the latter claiming there is no direct evidence to suggest that research would benefit if prescribing cannabis were no longer a criminal offence. Tony Moffat, its chief scientist, says that, if anything, regular users will have less incentive to join a clinical trial if they can obtain the drug from their doctors.

The report from the House of Lords points out that there is little cannabis research at UK universities and pharmaceutical companies. The Home Office has issued only 27 research licences over the past 25 years, and four licences this year.

The report's main recommendation is to move cannabis from schedule 1 of the Misuse of Drugs Regulations 1985 to schedule 2. A drug in schedule 1 cannot be grown, used or prescribed, except under a research licence. Schedule 2 drugs can be used on the instructions of doctors and dentists. Drugs in both classes can be used for research.

Although cannabis is a schedule 1 drug, its main psychoactive ingredient,  $\Delta^9$ -THC, is classified as a schedule 2 drug by the United Nations Convention on Psychotropic Substances, following advice from the World Health Organization that it has medical benefits.

Cannabis is the only schedule 1 drug of its kind that could be made legal for medicinal purposes by a change to UK law only. Any change to cannabis's remaining 60 constituent cannabinoids, which are all schedule 1 drugs, would need the assent of the UN convention.

Ehsan Masood

## India and Pakistan face more US sanctions

[NEW DELHI] A week after partially lifting trade sanctions against India and Pakistan, the US administration has tightened controls on technology exports to organizations believed to be involved in nuclear missile and military programmes.

Last Saturday (14 November), it named 250 entities in India and 90 in Pakistan for which US companies will require export licences to trade with. Most applications for licenses are expected to be denied.

Those singled out for sanctions in India include defence and atomic research agencies. Companies include the Fertilizer Corporation of India and Godrej & Boyce, which subcontracts from the Indian Space Research Organization, itself on the 'hit list'.

US officials in Delhi say the list will ease the burden on US exporters by "clarifying their responsibilities". The list is the largest release yet of US information on suspected nuclear and missiles proliferators to date.

The list is said to have been compiled by the Department of Defense, the State Department and the Central Intelligence Agency. It is divided into three categories: government agencies, government-affiliated and private companies, and military bodies.

The government's nuclear programme is denied all trade, but exports to government-affiliated bodies and private companies will be considered on a case-by-case basis. Military agencies will be denied export of items on a commerce commodity control list.

An Indian government spokesman described the move as "coercive and counterproductive". The Confederation of Indian Industries said publishing the list "would prove harmful to Indo-US business".

India plans to take the matter to the World Trade Organization (WTO). Commerce minister Ramakrishna Hegde said the US action was "inconsistent with WTO rules and regulations". **K. S. Jayaraman**



# Tight deadlines and data gaps fan fight on pesticide safety

Tony Reichhardt

**Farmers and chemical manufacturers are bracing themselves for a bitter fight with environmentalists in the coming months over new pesticide regulations. Both sides claim that science is on their side.**

[WASHINGTON] Moves by the US Environmental Protection Agency (EPA) to implement the Food Quality and Protection Act (FQPA), passed by Congress in 1996, are expected to reopen a battle about how much pesticide residue should be allowed in foods. The debate highlights the difficulty of setting public health policy in the absence of conclusive data about environmental threats.

The FQPA was meant to improve on the long-disputed Delaney clause, which had flatly banned any food additive found to cause cancer in animals. The new law substitutes a more flexible standard for food safety, demanding "reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue".

Based largely on recommendations contained in a 1993 report of the National Academy of Sciences, the FQPA also added an extra layer of protection for children. Pesticide 'tolerances' — the maximum amount of residue allowed in food — should be ten times lower for children than the levels deemed safe for adults, unless data show that children are not more susceptible.

The FQPA came with tight deadlines. One third of the nearly 10,000 uses for hundreds of pesticides had to be reassessed by August 1999, with another third reassessed by 2002, and the remaining third by 2006. The EPA has assigned priority to the 40 organophosphates, including malathion,

diazinon and Dursban (chlorpyrifos), which are widely used pesticides that target an insect's nervous system.

In the past three months, the EPA has published preliminary risk assessments for nearly half the pesticides in this group, but has not yet issued any revised tolerances. Preliminary analysis indicates that about half of the 40 organophosphates will require the additional three- to ten-fold safety factor to protect children.

## Lack of exposure data

Research on animals shows that organophosphates have harmful neurotoxic effects. But, although these toxicological data are strong, exposure data — the amount of pesticide residue present in food — are sketchy. Lacking reliable information, the EPA has assumed in the past that an entire field of crops is treated with the maximum legal amount of a pesticide, but farmers and pesticide manufacturers say this greatly exaggerates the amount of chemical applied.

The US Department of Agriculture, which collects information on pesticide usage, has so far been unable to produce a comprehensive, reliable database to settle the matter conclusively. Data on pesticide exposures in the home and in drinking water are even scantier, yet the FQPA requires the EPA to come up with a single 'aggregate' risk from all different routes. Methods for producing

this aggregate risk have yet to be developed.

The EPA has begun publishing guidelines as to how it will handle these and other issues related to the FQPA. These include: where to set safety thresholds when residue amounts are below the level of detection; what constitutes a "complete and reliable" database for assessing risks to children; and whether pesticides with a common method of toxicity might have cumulative effects.

Worried that the EPA will ban or restrict the use of pesticides based on assumptions rather than real-world data, farm groups and chemical manufacturers have lobbied for the agency to use its data 'call-in' authority to gather more information.

In a letter sent last month to Carol Browner, the head of the EPA, the American Farm Bureau Federation and American Crop Protection Association, which represents pesticide manufacturers, charged that, by not using this authority, the EPA is "needlessly jeopardizing tolerances for... products which could be proved to be safe if the necessary data were generated".

Environmentalists say the request for a data 'call-in' is a stalling tactic. An EPA official responsible for implementing the FQPA says only that "we'll look at all the information that is available to us", adding that industry-supplied data are of varying quality and relevance, and cannot always be accepted.

## A 'horrendous task'

Farmers and pesticide manufacturers counter that the law requires the use of real data, not models or estimates, and that piling one worst-case assumption onto another overestimates the threat to public health.

For example, the EPA sets its regulatory threshold for pesticides at a level 100 times below the dose at which no effect occurs in animals. Some pesticide companies have pushed for testing pesticides on humans because they believe that this uniformly applied safety margin is too conservative (see *Nature* 394, 515; 1998).

Faced with so many uncertainties, the EPA should slow down, say pesticide and farm groups. But, although acknowledging that there are holes in the data, environmental groups say the potential threat to children's health justifies action now.

More research could help to answer some important questions. But federal money for pesticide research has been scarce, and manufacturers rarely generate large amounts of data themselves unless forced to do so by regulatory deadlines.

The FQPA may provide that stimulus. Both sides agree that the law's vagueness — using terms like "reliable" information and "reasonable" harm — has left the EPA in a quandary, as it must write specific regulations based on that language. The time constraints alone, says one scientist, have created a "horrendous task" for the agency. □



Fine line: arguments still rage about how much pesticide can be safely applied to food crops.

AP/ELLIOTT MINOR

## Clinton 'troubled' by hybrid stem cell experiments

[WASHINGTON] President Bill Clinton has expressed strong concern about the implications of claims made last week that researchers have created hybrid, embryonic stem cells that are part human and part cow. The claims were made by researchers at Advanced Cell Technology, a Massachusetts-based biotechnology company, but details have not yet been published in the scientific literature.

Clinton asked the National Bioethics Advisory Commission to discuss the claims at its meeting in Miami earlier this week, and to report back to him immediately. "I am deeply troubled by this news of experiments involving the mingling of human and non-human species," he said in a letter to Harold Shapiro, the chairman of the commission.

The president also asked the commission to review US rules prohibiting the public financing of embryo research, in the light of the recent culturing and differentiation of human stem cells (see *Nature* 396, 104; 1998). Clinton hinted in his letter that the ban should be relaxed. He said that the benefits of such research were "hypothetical" in the past. But "it now appears that this

research may have real potential for treating such devastating illnesses as cancer, heart disease, diabetes and Parkinson's disease".

## Cold Spring Harbor to take PhD students

[LONDON] The Cold Spring Harbor Laboratory on Long Island, New York, is to offer a doctoral programme for the first time in its 108-year history.

The laboratory will enrol its first PhD students in its new school of biological sciences in September next year. Students will be expected to complete PhDs within four-and-a-half years, compared with the US average of between five and seven years.

The course is described as "deliberately interdisciplinary", and will focus on encouraging students to think critically and to develop good communications skills. This is an acknowledgement, says a laboratory spokesperson, that not all graduates will become academic researchers.

## Russian scientists reach salaries deal

[MOSCOW] The trade union that represents Russian scientific workers says that, for the first time in six months, it has helped the Russian cabinet to reach "some

understanding" of the problems faced by science, in particular the need to pay scientists' salaries.

According to Valery Sobolev, the chairman of the Russian Committee of Scientific Collectives, at a meeting with deputy prime minister Vladimir Bulgak at the end of October, "the government promised to follow the protocol we have signed, and we accepted that this is all the cabinet can do now to support scientists".

Given the cabinet's previous failure to fulfil its promises to scientists, the union has continued to picket the Ministry of Finance in Moscow. Union activists had been prepared for a national protest if the cabinet failed to pay salaries due by last Sunday (15 November). "This time the government faithfully followed its word," says Sobolev.

But Sobolev adds that the union will remain "in a state of one-week readiness for protest actions".

## European cash promotes collaborative networks

[LONDON] Research groups in Europe will be encouraged to form networks in eight new areas of research to be funded by the European Science Foundation in Strasbourg. The topics for collaboration include: the implications of the presence of extensive

microbial populations beneath the deep sea floor, the properties of silk, and the implications of European demographic trends on social services expenditure. Grants in the range of FF400,000 (US\$70,000) to FF600,000 will be available for each research network.

## Weiler takes space science job at NASA

[WASHINGTON] After flirting with outside candidates over the past few months, the US space agency NASA has appointed an insider, Edward Weiler, as its new associate administrator for space science.

Weiler, who has been acting head of the science office since Wesley Huntress left the job in late September, is currently science director for the agency's Origins programme to study galactic and planetary formation and search for evidence of life beyond Earth. An astrophysicist by education, Weiler has been NASA's programme scientist for the Hubble Space Telescope since 1979.

## Fund aims to launch life sciences companies

[MUNICH] Germany's largest life sciences venture capital fund was launched last week in the state of Hessen. The DM125 million

(US\$74 million) fund, Future Capital, is financed equally by the state government of Hessen and the Frankfurt-based pharmaceutical company Hoechst. It will support the foundation of life sciences and chemical companies in Hessen, as well as the promotion of existing high-technology companies.

Earlier this year, Hoechst increased its emphasis on genetics-based approaches to drug development (see *Nature* 393, 103; 1998). The company will have no say in funding decisions made by the venture capital fund. But Jürgen Dormann, chairman of the fund's board of management, hopes that the fund will promote collaboration between Hoechst and "world-class scientists involved in genetic engineering".

## Japanese space agency in overcharging claim

[TOKYO] The NEC Corporation has been accused of overcharging Japan's National Space Development Agency (NASDA) by more than ¥2.3 billion (US\$19.6 million) over five years.

The charges relate to work on the Japanese module of the International Space Station and the development of various satellites, including the communications and

broadcasting engineering satellite that is due for launch this year.

According to NASDA, which last week announced the preliminary results of an internal investigation, the electronics giant had allegedly been padding equipment and staff costs. NASDA says it plans to demand that the company returns the sum overcharged as soon as the exact amount has been determined.

## Parkes telescope notches up the 1,000th pulsar

[LONDON] Researchers using the 64-metre Parkes radio telescope in Australia have discovered the one-thousandth pulsar, 31 years after the first few were discovered in Cambridge. The pulsar was found using the telescope's new multibeam receiver, which comprises 13 beams that scan the sky simultaneously. As a result, pulsars are being discovered at a much faster rate than by any previous search.

Two hundred pulsars have been discovered in addition to the 800 that were known when the survey began a year ago. The receiver was jointly built by the Australia Telescope National Facility and the University of Manchester Nuffield Radio Astronomy Laboratories at Jodrell Bank.



# Clear need to act on global warming

*Sir* — David Victor<sup>1</sup> correctly points out reasons why M. I. Hoffert *et al.*<sup>2</sup> may have overstated the future need for carbon-free power, perhaps most significantly due to the vast potential for increasing energy efficiency. But I am troubled by Victor's conclusion that "taking action on global warming is akin to buying insurance with an unknown premium against unknown hazards" and that "what to do is unclear".

This neglects two important issues. First, there are widely accepted 'no regrets' strategies that improve the economy and help solve other environmental problems while simultaneously providing insurance against climate change. Such strategies, in particular improving energy efficiency, are often achieved at no net cost (or even with net savings)<sup>3,4</sup>. So the 'premium' is zero, or

close to zero, and the hazards, although not precisely known, are insured against while providing other benefits.

Second, recent work on decision-making under uncertain conditions provides guidance for taking action on global warming. R. J. Lempert *et al.*<sup>5</sup>, for example, have modelled an adaptive strategy that makes midcourse corrections based on observations of the climate and economic systems. They find that such a strategy helps avoid the pitfalls of policies based on best estimates. Without knowing with certainty the climate sensitivity, the damages that will result from climate change, or the rate of technological innovation, we cannot choose the optimal policy and should instead create a strategy that allows for adaptation based on new learning. Problems for the

present thus include developing better options for massive greenhouse-gas reductions than are currently available and determining what observations ought to trigger their implementation.

"What to do" is not so unclear as Victor implies. Aggressive and immediate implementation of 'no regrets' efficiency improvements plus more research on carbon-free energy sources seems a responsible and conservative approach.

**Susan Hassol**

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## Region-based citation bias in science

*Sir* — R. M. May<sup>1</sup> ranked countries according to their share of articles and the share of citations the articles received. As the ratio of the two indicators is less than unity in France, Germany, Italy and Japan, he claimed a lower-than-average quality of their publications. This might be the case if most of their papers were published in journals with low impact factor (IF); instead, however, we think that most papers receive less citations than they deserve even if they appear in journals with good IF.

To this end we have considered 206 international journals in 14 environment-related categories of the Institute for Scientific Information database and have analysed the citations received by Italian scientists. For each category, we have computed the Italian papers' IF as the weighted sum of the journal IFs according to the number of Italian papers in each journal. In 12 cases out of the 14 (94% of the journals), the Italian papers' IF is either equal to, or significantly higher than, the mean IF of the journals in the respective category. This shows that Italian scientists tend to publish in high-quality journals.

We then compared the number of citations received by each Italian article with the expected citation rate (XCR), that is, the average citation per paper based on the journal title, year of publication and type of document. As suggested by Barreto<sup>2</sup> and May<sup>3</sup> himself, if there were no bias, the number of citations per article would not be significantly higher or lower than the XCR. However, this is not so (see Table 1).

**Table 1 Statistics of undercitation for Italian environmental scientists**

| IF class | No. of papers | Proportion undercited | Significance, P |
|----------|---------------|-----------------------|-----------------|
| <0.4     | 314           | 0.67                  | <0.005          |
| 0.4–0.6  | 519           | 0.68                  | <0.0001         |
| 0.6–0.8  | 939           | 0.69                  | <0.0001         |
| 0.8–1.2  | 1,032         | 0.74                  | <0.0001         |
| 1.2–2    | 1,099         | 0.72                  | <0.0001         |
| 2–4      | 364           | 0.78                  | <0.0001         |
| 4–28     | 45            | 0.84                  | <0.0001         |

The number of papers in each IF class are shown along with the proportion of undercited papers (that is, XCR greater than total citations). Significance, Wilcoxon test.

A further clue to bias comes from analysing the scientific productivity at Ispra (Italy), the Joint Research Centre of the European Commission comprising mostly non-Italian environmental scientists. Although the IF is not higher than average, the number of citations significantly exceeds the XCR (Wilcoxon matched-pairs test,  $P < 0.05$ ).

It is difficult to pinpoint the causes of the bias highlighted by our analysis. There are many motivations behind the citation process — for example, acknowledging pioneers, giving credit to related work and providing background reading. We believe that, while the process of accepting a paper on a journal is reasonably objective, with several referees commenting on the quality and merits of the paper regardless of its country of origin, that of citing a paper is more subjective and certainly more open to considerations of career and funding opportunities. As English-speaking countries produce 49.2% of the world's papers<sup>1</sup>, they have a dominant position in the scientific world. Not citing their scientists' work would produce negative feedback, while the converse would not. In

other words, awareness of the citation game can influence the choice of citations.

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## Subtle error on sea floor

*Sir* — In the otherwise excellent article<sup>1</sup> "Subtle minds and mid-ocean ridges", it is erroneously stated that Earth's fastest seafloor spreading occurs in the 15°–18° S East Pacific Rise "MELT" area.

Spreading rates are known to increase further south along this ridge<sup>2,3</sup>, although exactly where the fastest spreading occurs is complicated by the presence of duelling propagating rifts and microplates, where total plate separation is accommodated on dual active spreading centres. The fastest spreading known today (some 149 mm yr<sup>-1</sup>) thus occurs near 31° S, south of the large-scale duelling propagators between the Easter and Juan Fernandez microplates<sup>4</sup>.

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# The greening of the green revolution

David Tilman

**In comparison with conventional, high-intensity agricultural methods, 'organic' alternatives can improve soil fertility and have fewer detrimental effects on the environment. These alternatives can also produce equivalent crop yields to conventional methods.**

It is not clear which are greater — the successes of modern high-intensity agriculture, or its shortcomings. The successes are immense. Because of the green revolution, agriculture has met the food needs of most of the world's population even as the population doubled during the past four decades. But there has been a price to pay, and it includes contamination of groundwaters, release of greenhouse gases, loss of crop genetic diversity and eutrophication of rivers, streams, lakes and coastal marine ecosystems (contamination by organic and inorganic nutrients that cause oxygen depletion, spread of toxic species and changes in the structure of aquatic food webs)<sup>1,2</sup>. It is unclear whether high-intensity agriculture can be sustained, because of the loss of soil fertility, the erosion of soil, the increased incidence of crop and livestock diseases, and the high energy and chemical inputs associated with it<sup>1,3</sup>. The search is on for practices that can provide sustainable yields, preferably comparable to those of high-intensity agriculture but with fewer environmental costs<sup>1</sup>.

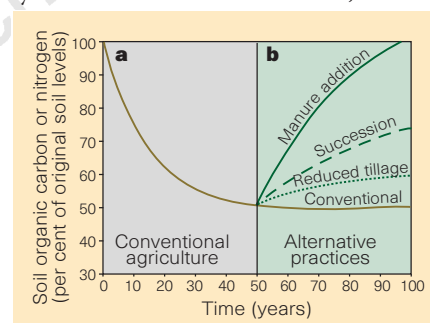
For millennia, farmers countered the loss of soil fertility caused by agriculture (Fig. 1) by manuring fields, by alternating crops that increase soil fertility (such as legumes, which 'fix' atmospheric nitrogen into organic compounds and so add nitrogen-containing compounds to the soil) with other crops, and by abandoning fields and allowing them to be taken over gradually by natural vegetation (succession). This changed with the advent of the green revolution.

A hallmark of high-intensity agriculture is its dependence on pesticides and chemical fertilizers, especially those containing nitrogen. Since 1960 the worldwide rate of application of nitrogen fertilizers has increased by seven times<sup>2</sup>, and now exceeds  $7 \times 10^7$  tonnes of nitrogen per year. Inputs from humans now equal all natural inputs to the nitrogen cycle and are seriously affecting terrestrial, freshwater and marine ecosystems<sup>2</sup>, because half to two-thirds of nitrogen fertilizers enter these non-agricultural ecosystems.

On page 262 of this issue<sup>4</sup>, Drinkwater, Wagoner and Sarrantonio report two alternative practices for growing maize that maintain yields while increasing soil fertility

and decreasing losses of nitrogen by leaching. This advance is not based on a miracle of technology but is a lesson from agriculture's past that may presage its future.

In Drinkwater and colleagues' conventional, high-intensity system, pesticides and mineral nitrogen fertilizer were applied to a maize/soybean crop rotation just as on typical farms. Two 'organic' alternatives represented partial returns to traditional agriculture, and neither synthetic fertilizers nor pesticides were used. One of these alternatives was a manure-based system in which grasses and legumes, grown as part of a high-diversity crop rotation, were fed to cattle. The resulting manure provided nitrogen for periodic maize production. The other system did not include livestock; instead,



**Figure 1 Typical effects of different agricultural practices on the total organic carbon or nitrogen content of soil. a, Over about 50 years, conventional agricultural fields that are tilled and then fertilized with mineral nitrogen, phosphorus and potassium (NPK) fertilizer lose 50–65% of pre-agricultural amounts of soil carbon and nitrogen<sup>1</sup>. b, The effects of different practices on recovery of soil fertility after 50 years of tilling. Practices that reduce the amount of tillage of soils can lead to accumulation of about 20% of the lost carbon or nitrogen<sup>1</sup>. Succession here refers to the process by which abandoned fields are gradually invaded by successive populations of native vegetation and animals, eventually reaching a stable 'climax' community structure. We have found that it takes about 200 years of natural succession for fields to recover pre-agricultural carbon and nitrogen levels (J. Knops and D. Tilman, manuscript in preparation). The addition of manure can double soil carbon or nitrogen levels in about 40 years<sup>7,9</sup>.**

nitrogen fixed by a variety of legumes was incorporated into soil as the source of nitrogen for maize.

Amazingly, ten-year-average maize yields differed by less than 1% among the three cropping systems, which Drinkwater *et al.* say were nearly equally profitable. The manure system, though, had significant advantages. Soil organic matter and nitrogen content — measures of soil fertility — increased markedly in the manure system (and, to a lesser degree, in the legume system), but were unchanged or declined in the conventional system. Moreover, the conventional system had greater environmental impacts — 60% more nitrate was leached into groundwater over a five-year period than in the manure or legume systems.

Why were the organic methods superior to conventional, high-intensity agriculture? The answer is not yet known, but two possibilities stand out. First, when fertilizing, timing is crucial<sup>5</sup>. The nitrogen pulse from a single application of mineral fertilizer can cause soil nitrate concentrations to greatly exceed plant needs. The unconsumed nutrients are susceptible to loss by leaching and denitrification. In contrast, the organic methods supply nitrogen in organic forms that gradually release mineral nitrogen, perhaps better synchronizing nutrient availability with plant needs.

Second, although equivalent amounts of nitrogen and organic carbon were added to the soil in all three systems, the manure system included a higher proportion and greater diversity of recalcitrant (that is, slowly biodegradable) organic compounds than the conventional system. This may have caused carbon and nitrogen to accumulate in the manure system, minimizing leaching losses. Indeed, models of soil carbon and nitrogen dynamics predict such accumulation when fields are manured<sup>6,7</sup> (Fig. 1).

Drinkwater and colleagues' results may seem astounding, or even suspect, given the widespread use of chemical fertilizers. They are not. In the Broadbalk experiment (Fig. 2, overleaf), at the Rothamsted Experimental Station in the United Kingdom, which has been running for more than 150 years, wheat yields have averaged 3.45 tonnes per hectare on manured plots compared with 3.40 tonnes per hectare on plots receiving complete nitrogen, phosphorus and potassium (NPK) fertilizer<sup>8</sup>. Moreover, soil organic matter and soil total nitrogen levels increased by about 120% over 150 years in the manured plots (Fig. 1), but by only about 20% in the NPK plots<sup>7,9</sup>. Such carbon stores might represent an underappreciated sink for global carbon.

The intensification of agriculture has broken what was once the tight, local recycling of nutrients on individual farms. Indeed, the green revolution and the large-scale livestock operations that have come with it are remi-



Figure 2 Field work: experiments on fertilizer regimes have run at Rothamsted since 1843.

niscient of the early stages of the industrial revolution, when inefficient factories polluted without restriction. The US Environmental Protection Agency estimates that, in the United States alone, livestock operations generate about  $10^9$  tonnes of manure per year, much of it in large-scale operations in which up to a million or more animals are housed in close quarters. These concentrated sources of manure are often too far from farms to be economically transported to them, or are applied at inappropriately high rates or at incorrect times, or are released into waterways without removing nitrogen and phosphorus. This has created an open nitrogen cycle that is rapidly degrading many other ecosystems<sup>2</sup>. Sustainable and productive ecosystems have tight internal cycling of nutrients, a lesson that agriculture must learn.

The results of Drinkwater and colleagues<sup>4</sup> are a step in the right direction. What may lead to further progress? The green revolution turned developments in crop genetics, inexpensive pesticides and fertilizers, and mechanization into greater yields. Further advances, such as precision agriculture, in which fertilizer application rates and timing are adjusted differentially across a field to meet crop needs, will increase agricultural efficiency and decrease adverse effects on the environment. However, a greener revolution is also needed — a revolution that incorporates accumulated knowledge of ecological processes and feedbacks, disease dynamics, soil processes and microbial ecology. Experiments such as those of Drinkwater *et al.* need to be combined with studies of both the mechanisms controlling soil organic matter and nitrogen dynamics<sup>6,7,9</sup>, and the dynamics of crop nutritional needs.

The principles of ecology, epidemiology, evolution, microbiology and soil science operate in agroecosystems as well as in natural ecosystems. Although the owners of the businesses were probably shocked, I doubt if epidemiologists were surprised that Hong Kong chicken operations, housing up to a million genetically similar chickens, were susceptible to a rapid and devastating out-

break of disease last year. When those running massive livestock operations realize that chronic disease and catastrophic epidemics are the expected result of high densities and low diversity, and when society restricts the release of pollutants from such operations, it may again be profitable for individual farms, or neighbourhood consortia, to have mixed cropping and livestock operations tied together in a system that gives an efficient, sustainable, locally closed nitrogen cycle.

#### Earth science

## Prospecting for hotspot roots

Cecily J. Wolfe

Oceanic islands commonly form linear chains with progressively increasing age away from the active region, reflecting nearly stationary 'hotspots' beneath the moving lithospheric plate. These hotspots are believed to be produced by upwelling mantle plumes formed from instabilities at a hot thermal boundary layer in the Earth's mantle, but the depth of origin of mantle plumes has long been debated.

On pages 251 and 255 of this issue, the seismologists Helmberger *et al.*<sup>1</sup> and Russell *et al.*<sup>2</sup> propose that they have identified the roots of the Iceland and Hawaiian hotspots at the core-mantle boundary, some 2,900 km beneath the Earth's surface. Their results are notable because although some hotspots may reflect shallow melting anomalies rather than a deeply rooted mantle plume, Iceland and Hawaii are the two classic hotspots that could plausibly represent continuing upwelling from the core-mantle boundary. These hotspots are long-lived, erupt large volumes of magma, and have distinctive geochemical signatures (in particular a high ratio of  $^3\text{He}$  to  $^4\text{He}$ , which is interpreted as  $^3\text{He}$  from an undegassed region of the mantle).

For several years, seismologists brought little insight into resolving plume character-

No other activity has transformed humanity, and the Earth, as much as agriculture<sup>10</sup>, but the environmental effects of high-intensity farming increasingly haunt us. In a small world awash with the waste products of humanity, there is a great need to find new approaches to agriculture. □

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istics at depth, and the most exciting research on mantle plumes was left to geochemists, who studied ocean island basalts and revealed that hotspots sample isotopically and chemically distinct reservoirs in the Earth's mantle<sup>3</sup>. But seismologists are beginning to catch up at an alarming pace — within the past few years we have had images of a mantle plume conduit in the upper mantle<sup>4</sup> and transition zone (410–660-km depth)<sup>5</sup>, and the discovery of features in the lowermost 250 km of the mantle that may pinpoint the source region for mantle plumes<sup>1,2,6</sup>. The explosion in our knowledge of the lowermost mantle<sup>6</sup> is credited to detailed analyses of seismic waveforms for a variety of secondary phases, improvements in instrumentation, and the free and easy access that seismologists are typically given to global and regional data sets.

Helmberger *et al.*<sup>1</sup> show that a feature known as an 'ultra-low velocity zone' can be detected at the core-mantle boundary beneath Iceland, and they argue that this anomalous area may reflect the hot, partially molten source of the Iceland mantle plume (Fig. 1). Garnero, Grand and Helmberger<sup>7</sup> originally pointed out the likely existence of such zones — now defined as a 5–40-km thick layer at the base of the mantle with P- and S-wave seismic velocity reductions of



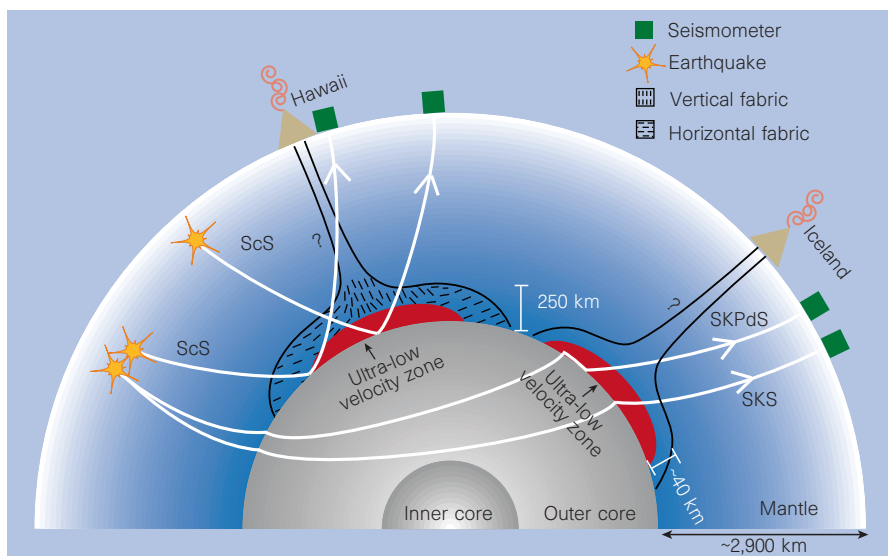


Figure 1 Summary of the evidence from which Helmberger *et al.*<sup>1</sup> and Russell *et al.*<sup>2</sup> infer that the origins of the Iceland and Hawaii mantle plumes lie at the core–mantle boundary. At the core–mantle boundary beneath Iceland, Helmberger *et al.*<sup>1</sup> observe a 40-km thick ultra-low velocity zone from the delay and amplification of the phase known as SKPdS, which samples the ultra-low velocity zone, with respect to the phase SKS. At the core–mantle boundary southeast of Hawaii, Russell *et al.*<sup>2</sup> observe that the anisotropy of ScS phases indicates a change from horizontal fabric to vertical fabric, which they attribute to plume upwelling. These features may reflect the hot, upwelling roots of the Icelandic and Hawaiian plumes.

10% or more — from the delay and amplification of the core phase known as SKPdS with respect to the core phase SKS. Since then, seismologists have been busy mapping the spatial distribution and characteristics of ultra-low velocity zones and considering whether they mark the roots of mantle plumes.

Although the global picture is still incomplete, the pattern documented so far indicates that such zones are correlated with hotspot locations<sup>8</sup>, although a zone less than 5 km thick would be undetectable by these methods. It has been suggested that the severe drops in seismic velocities reflect the presence of partial melt, which should induce a three times greater reduction in S-wave than in P-wave velocity. The preferred model of Helmberger *et al.* contains velocity reductions of 10% for P-waves and 30% for S-waves, consistent with the hypothesis of partial melt; the S-wave velocity drop has some tradeoffs with other parameters, however, including the density and thickness of the layer, and layer shape.

Russell *et al.*<sup>2</sup> employ a different set of methods, analysing ScS and S phases (ScS is a shear wave that bounces off the core–mantle boundary) to detect a region of lateral gradients in velocity and anisotropy in the lowermost mantle. They suggest that this feature is a signature of the source region of the Hawaiian hotspot.

The pattern of ScS anisotropy is unusual. Previous measurements of detectable anisotropy in the lowermost 250 km of the mantle display horizontally polarized (SH) waves arriving ahead of vertically polarized

(SV) waves. These observations have been explained by a horizontally layered media or the presence of horizontally elongated melt inclusions<sup>9</sup>. Russell *et al.* find that this pattern changes to SV arriving ahead of SH in a localized region beneath the central Pacific, which they attribute to plume upwelling producing a vertical fabric. But anisotropy at the core–mantle boundary remains poorly mapped. So one cannot yet judge whether such changes in anisotropy will be found to be correlated with hotspots and turn out to be diagnostic of plume roots.

## Vascular biology

# Old-timer makes a comeback

Paul M. Vanhoutte

When, in 1987, the endothelium-derived relaxing factor discovered by Robert Furchgott was shown to be nitric oxide (NO)<sup>1</sup>, I asked<sup>2</sup> whether all endothelium-dependent relaxations might be explained by the release of this volatile mediator. It has since become clear (Fig. 1, overleaf) that a local autacoid, prostacyclin, as well as at least one other diffusible dilator — endothelium-derived hyperpolarizing factor (EDHF)<sup>3,4</sup> — can also contribute to certain endothelium-dependent relaxations. Now, on page 269 of this issue, Edwards *et al.*<sup>5</sup> propose an identity for EDHF. They suggest that, in small arteries of the rat at least, potassium ions (K<sup>+</sup>) are responsible for the hyperpolarization caused by acetylcholine in the presence (but not the

Finally, the interpretations of both studies remain open to debate, because geophysicists simply do not know the location of the Iceland and Hawaiian plume conduits all the way through the mantle, as it is not clear how rising mantle plumes may be tilted by mantle convection. It would be very helpful if seismologists could map a hotspot from its surface expression to the core–mantle boundary. That is no easy matter, however — although tomographic images reveal subducting slabs sometimes extending throughout the mantle<sup>10</sup>, it will be more difficult to image the trails of narrow hotspots as these features are smaller and the signal from slow anomalies will be reduced by diffraction. For the time being, Earth scientists will have to be content with the wealth of new information being uncovered by seismology at the core–mantle boundary. □

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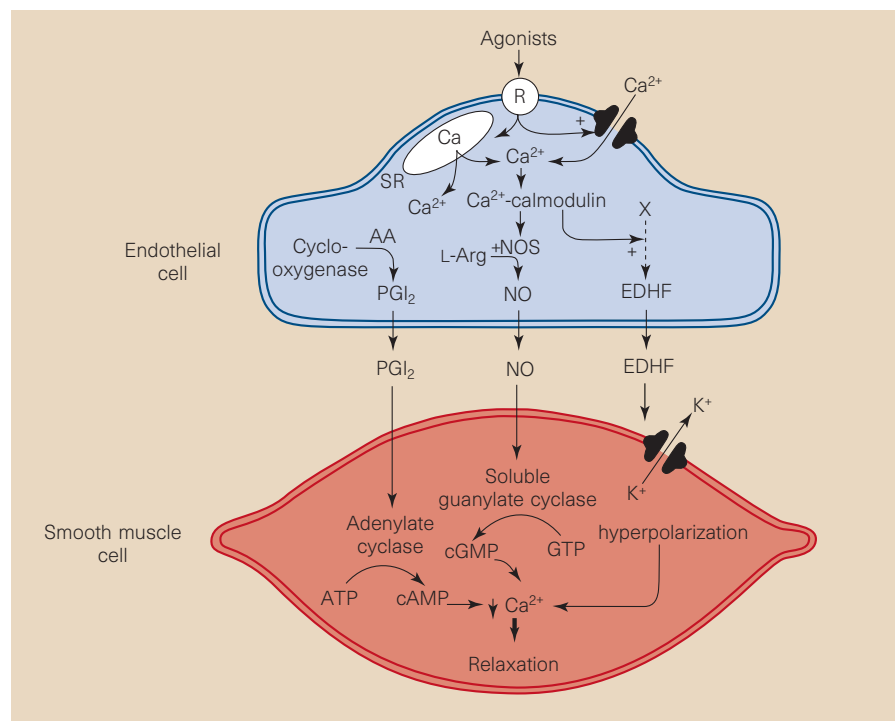
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absence) of endothelial cells.

The nitric oxide field has unfolded in recent years, not only because the mediator was identified<sup>1</sup>, but also owing to the discovery of drugs, such as L-NMMA, that selectively inhibit its formation<sup>6</sup>. Studies of EDHF, on the other hand, have been hampered by persisting controversy over its real identity (Fig. 2), and the fact that the substances proposed to inhibit its formation (such as calmodulin antagonists and P-450 inhibitors) are notoriously nonselective. Hence the suggestion by Rudi Busse to rename EDHF the ‘eternally deceptive hyperpolarizing factor’.

Edwards *et al.* now suggest both an identity (K<sup>+</sup>) for, and antagonists (ouabain and barium ions) against, EDHF. In proposing



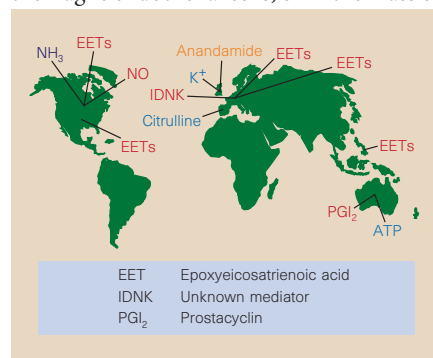
**Figure 1** Relaxation of vascular smooth muscle cells by diffusible vasodilator substances from endothelial cells. Prostacyclin ( $\text{PGI}_2$ ) activates adenylate cyclase, leading to increased production of cyclic AMP (cAMP). Nitric oxide (NO) activates soluble guanylate cyclase, yielding increased levels of cyclic GMP (cGMP). Endothelium-derived hyperpolarizing factor (EDHF), which Edwards *et al.*<sup>5</sup> now propose to be  $\text{K}^+$ , causes  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels in vascular smooth muscle cells to open, leading to their hyperpolarization. The three mediators are not always equally important — NO may predominate in large arteries, whereas EDHF takes over in smaller blood vessels or in large arteries when the release of NO is curtailed. AA, arachidonic acid; NOS, NO synthase; L-Arg, L-arginine; R, membrane receptor; SR, sarcoplasmic reticulum; X, unknown precursor.

$\text{K}^+$  as EDHF, they ensure the comeback of an old-timer in local regulation of vascular diameter<sup>7</sup>. Their results could explain earlier work showing that  $\text{Na}^+$ – $\text{K}^+$  exchanges contribute to the responses of isolated arteries to acetylcholine<sup>8</sup>, and the authors revamp the image of ouabain as a remarkable inhibitor that blocks the action of EDHF<sup>3</sup> but not that of  $\text{NO}^{9,10}$ . But there are still questions as to whether ouabain is selective, and whether its effect is truly due to inhibition of  $\text{Na}^+$ – $\text{K}^+$  exchanges, particularly in rat tissues. Another problem with ouabain, not to mention barium, is that use *in vivo* will be extremely limited. So, we still await a molecule that inhibits the production or action of EDHF with the same power and selectivity as L-NMMA.

Before we celebrate the identification of  $\text{K}^+$  as the universal EDHF, there are several other questions to be answered. First, can endothelial cells release enough  $\text{K}^+$  to be transferred across the existing diffusion barriers, such as the basal membrane and elastic laminae, on its way to the smooth muscle? How much  $\text{K}^+$  is involved, and can it be generated by the thin monolayer of endothelial cells? And is this plausible in thick-walled arteries such as the human coronaries<sup>11</sup>? Edwards *et al.* briefly report impalement experiments with  $\text{K}^+$ -sensitive electrodes,

but one must take their word for this (as they provide no real clues to allow others to reproduce their measurements), and accept that they have managed the *tour de force* of successfully inserting an electrode into the space between the endothelium and the media.

Second, is the surge in  $\text{K}^+$  that Edwards *et al.* measure due to opening of  $\text{K}^+$  channels in the fragile endothelial cells, or in the mass of



**Figure 2** Geography of research into endothelium-derived hyperpolarizing factors (EDHFs). Blue names, candidate EDHFs proposed by one research group so far; red names, EDHFs under investigation, with reasonable data to back them up; orange names, candidate EDHFs supported by one research group but not by others; mauve names, candidate EDHFs that did not stand the test of time.

smooth muscle that surrounds them (Fig. 1)? Likewise, given that the main function of a hyperpolarizing factor should be to hyperpolarize, how do we interpret the dissociation of the effects of ouabain/barium on the response to acetylcholine, whereby hyperpolarization is abolished yet relaxation is hardly affected? Does this mean that the endothelium-dependent hyperpolarization is an epiphenomenon? And, if so, how does this fit with the fact that the search for EDHF was initiated to explain endothelium-dependent relaxations that persist in the absence of NO? Finally,  $\text{K}^+$  has long been known to contribute to metabolic hyperaemia<sup>7</sup>, so is endothelium-dependent hyperpolarization yet another unique role for it, or is  $\text{K}^+$  only one of many EDHFs (Fig. 2)?

Max Planck once said “A new scientific truth does not triumph by convincing its opponents and making them see the light, but rather because its opponents eventually die, and a new generation grows up that is familiar with it”. Most of the players in the EDHF field are still quite young, so answering the remaining questions seems to be the only way to bring researchers together. These queries do not detract from the elegance of Edwards and colleagues’ study, nor do they question the validity of their observations — rats and their arteries do not lie. But, until we have proper answers, we cannot extrapolate to the entire vascular system. This is not a matter of disbelief, but of scientific caution.

When commenting<sup>2</sup> on the demonstration by Moncada and colleagues that the Furchgott endothelium-derived relaxing factor is indeed NO, I marvelled at the ingenuity of endothelial cells, which use very simple molecules rather than complex peptides. If Edwards *et al.* are right, these endothelial cells are even more primitive than I thought in 1987, because they use monovalent cations for intercellular signalling. Commentaries on this year’s Nobel prize for physiology or medicine<sup>12</sup> sought an amusing link between nitric oxide and the nitroglycerine that produced the money to fund the award in Nobel’s days. If EDHF is  $\text{K}^+$  (at least in certain arteries), and if its action is really blocked by cardiac glycosides<sup>3,8–10</sup>, this endothelial mediator would have a much more impressive history dating back to William Withering and the foxglove — the true beginning of modern pharmacology. And EDHF would then stand not for ‘eternally deceptive’, but for the ‘ever distinguished hyperpolarizing factor’.

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#### 100 YEARS AGO

A few particulars of Mr. Nikola Tesla's new method of electric power transmission are given in the current number of the *Electrical Review*. From the article it appears that the invention consists in transmitting electrical power without the employment of metallic line conductors, by taking advantage of the conductivity of the rarefied air existing in the upper regions of the earth's atmosphere. ... Heretofore, it has been possible, by means of the apparatus at command, to produce only moderate electrical pressures, and even these with considerable risks and difficulties. Mr Tesla, however, claims that he has devised means whereby he is enabled to generate, with safety and ease, electrical pressures measured by hundreds of thousands, and even by millions of volts.

I think perhaps it may be worth noting that apple-blossom was gathered in the neighbourhood of Exeter last week. Still more remarkable is the fact that a second crop of apples has made fair progress ... [S]ome "Red Quaranders" have been gathered, nearly the size of walnuts. Two of these, now somewhat shrivelled, are enclosed.

From *Nature* 17 November 1898.

#### 50 YEARS AGO

It is becoming more and more clear to most people connected with British Industry that the way out of the present economic crisis will not be by the introduction of new machines. ... Among the ways which have been and are being increasingly used to add to overall efficiency is what industry has come to call 'education and training'. Simple as this may sound, it is surprising that, even in 'enlightened' firms, the process of providing a man with specific training to do a job is often regarded as something which hinders rather than helps production. Engineers, of course, have to be trained; so have chemists. They may even need a four-year preliminary course before their training starts. But the semi-skilled and unskilled workers can pick up their jobs by being put alongside Joe and Tom. In the Services men are given their training before being assigned to a unit; in industry many men and women have to learn their jobs by picking up all that cannot be concealed from them.

From *Nature* 20 November 1948.

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#### Biogeography

## Competition exposed by knight?

Peter R. Grant

"To do science is to search for repeated patterns, not simply to accumulate facts" — so ran the famous opening sentence of Robert MacArthur's *Geographical Ecology*<sup>1</sup>. There is no better exemplar than Jared Diamond's attempt, three years later, to extract pattern and meaning from a mass of biogeographical data on bird species on islands around New Guinea<sup>2</sup>. The data and Diamond's interpretation of them have been controversial, but they have now been re-evaluated by Sanderson, Moulton and Selfridge, writing in *Oecologia*<sup>3</sup>.

One pattern that stood out in Diamond's analysis was a lack of co-occurrence of certain bird species, which nonetheless have interdigitating geographical distributions — that is, the species occur throughout the archipelago. He interpreted this pattern as evidence of diffusely acting competitive exclusion, by which he meant species were excluded from certain islands by the combined competitive influence of a set of others; he therefore built competition into a set of rules that appeared to have governed the assembly of communities of bird species on islands. This touched off a long and sometimes acrimonious debate about the statistical evidence for competition in nature<sup>4–6</sup>. Sanderson *et al.*<sup>3</sup> report how they have overcome statistical limitations of previous analyses. Their results provide support for Diamond's original interpretations, while leaving some intriguing issues open.

Three questions have been asked of Diamond's work. Was the pattern identified correctly? If so, was the causal process inferred correctly? And how general are the rules? The first is pivotal. Connor and Simberloff<sup>4</sup> addressed it by employing a statistical method that yields a distribution of species occurrences expected by chance. Ten times they randomly reassigned the 56 Vanuatu (New Hebrides) bird species to the 28 islands in the archipelago, while preserving unchanged the observed number of species per island and the number of islands per species. The small number of reassignments, constrained by the nature of the data, were used to generate a 'null' distribution of species occurring together on no islands (so-called checkerboards), one island, two islands, three islands and so on. Connor and

Simberloff then compared the observed distribution with the computed one, found close agreement with a  $\chi^2$  test, and concluded there was no evidence for any systematic structuring process such as interspecific competition.

The debate has focused on how the null model should be constructed<sup>5,6</sup>. If competition between species has indeed structured communities, the random scrambling of species occurrences might defeat the purpose of statistically detecting it<sup>7,8</sup>, so it is crucial to construct the null model in a satisfactory way<sup>5,7–9</sup>. The random scrambling technique has been avoided altogether by Sanderson and colleagues<sup>3</sup>. To appreciate their alternative it is helpful to think in terms of a matrix, with the names of islands at the top of the columns and individual species listed at the left margin of the rows. In the matrix itself a species does (1) or does not (0) occur on a particular island.

To produce a genuinely null model Sanderson *et al.* used a recursive algorithm, commonly used in computer science, known as the knight's tour. As in chess, the knight moves in an L pattern. The goal of the tour is to touch each cell of a matrix once and only once. When thwarted in forward moves it backtracks, reorients and moves forward again. Starting with a truly null matrix of 0s, and operating by random placement of species and the knight's rule of movement, they sequentially filled the matrix with species (1s) until all observed column and row totals were obtained. No less than 5,000 unique matrices were generated by this procedure, giving a distribution of frequencies of species co-occurrences to compare with the actual ones.

The result? Species pairs never occurring together are as frequent as expected by chance, but those occurring together twice and only twice are improbably rare; they fall outside the computed 99% confidence limits. Improbable rarity supports the competition hypothesis<sup>2</sup> because it implies certain combinations of species can coexist only under rare circumstances. But other hypotheses invoking predation, parasitism, hybridization, habitat requirements, different colonization routes, time lags in the dispersal of species throughout the archipelago and so forth are not excluded.



Null models are used to assess departures from randomness in other fields such as palaeobiology<sup>10</sup> and the reconstruction of phylogeny<sup>11</sup>, so this new analytical procedure will be useful beyond community ecology. Some interlocking statistical and biological concerns remain, however, centred on the problem of interdependencies.

For example, in the Vanuatu analysis pairs occurring nine and only nine times, like the twos, are improbably rare, but pairs occurring ten and only ten times are improbably common. These opposite directions in deviation of 'neighbours' in the frequency distribution (and there are others) hint at further structure in the data, structure that may be statistical<sup>12,13</sup> or biological. The source of that structure might lie in the full set of 56 bird species being analysed together, rather than just guilds of potential competitors, and in the artificiality (and convenience) of holding the number of islands per species constant, for which there is no biological theory; in contrast there is good biogeographical theory<sup>14</sup> to justify holding the number of species per island constant in the null modelling.

This raises the issue of how a biologically realistic null model can be constructed for communities of species in an archipelago when the source area and set of species cannot be specified; and of how sequential colonization and local adaptation, geography and history<sup>15</sup>, can be adequately built into the null model. In fact, how much biology

should be incorporated into the null model?

Here we have echoes of a major issue in population genetics — whether variation is maintained by random drift or selection. We are left, as Diamond<sup>2</sup> was, with a challenging problem, that of determining the relative influences of chance and necessity (systematic forces, such as competition) in the assembly of biological communities through time. □

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## Photonics

# An atomic dimmer switch

Philip H. Bucksbaum

On page 239 of this issue<sup>1</sup>, Doron Meshulach and Yaron Silberberg of the Weizmann Institute show how atoms can be made to absorb light more or less readily, or not at all, simply by controlling the phase of light shone at them. This joins a growing list of new 'coherent control' methods for manipulating the internal quantum dynamics of atoms and molecules using the coherence properties of light, rather than its intensity or colour.

All atoms and molecules can absorb light. The absorption usually occurs for light that has a frequency  $\nu$  at resonance — that is where the energy of a single photon in the light beam,  $h\nu$ , equals the energy difference between the ground state and an excited quantum state of the system. But when an atom is subjected to intense light, such as that produced by a laser, nonlinear absorption is also possible. In nonlinear absorption, two or more photons pool their energy to excite the atom, and the sum of the photon energies equals the excited-state energy difference. The photons must not only have the right

total energy, they must also arrive at the target at nearly the same time, so nonlinear optical effects are usually stronger when the light is compressed into a short, intense pulse. This is the principle behind several practical devices in lasers such as harmonic frequency converters.

One reason that the arrangement of photons is important in a nonlinear process is quantum interference. When a quantum dynamical process can take more than one path, interference occurs between the different possible routes. The interference may be destructive or constructive, slowing or has-

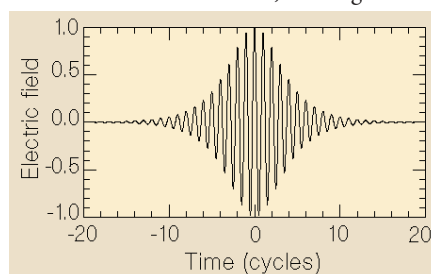


Figure 1 The electric field of a short laser pulse.

tening the process. An example is the two-photon absorption experiment described by Meshulach and Silberberg: if the photons are both present at the same time, then the absorption may take two paths, corresponding to absorption of photon 1 first, followed by photon 2, or the other way around. Coherent control provides a way to adjust the quantum interferences between these different paths, affecting the rate of the whole process.

This is easier to understand if we stop talking about photons and consider the light as a classical electromagnetic wave. The light pulse is a travelling wave with frequency  $\nu$  and electric field amplitude  $A$ , so that an atom will experience the light as an oscillating electric field  $E = A \cos(2\pi\nu t)$  where  $t$  is time. Figure 1 is a picture of the electric field versus time for a typical short laser pulse. The turn-on and turn-off create a frequency spread, shown in the spectral density of this pulse given by its Fourier transform (Fig. 2a).

An atom with a resonance at any frequency in this spectrum can be excited by absorbing a single photon of light from the pulse. Each frequency present can have different phases as well as different amplitudes — that is, the light with frequency  $\nu_1$  might be a sine wave,  $\sin(2\pi\nu_1 t)$  while the light with  $\nu_2$  might be a cosine wave  $\cos(2\pi\nu_2 t)$  or any intermediate phase. In Fig. 1, all the different frequencies happen to be cosine waves.

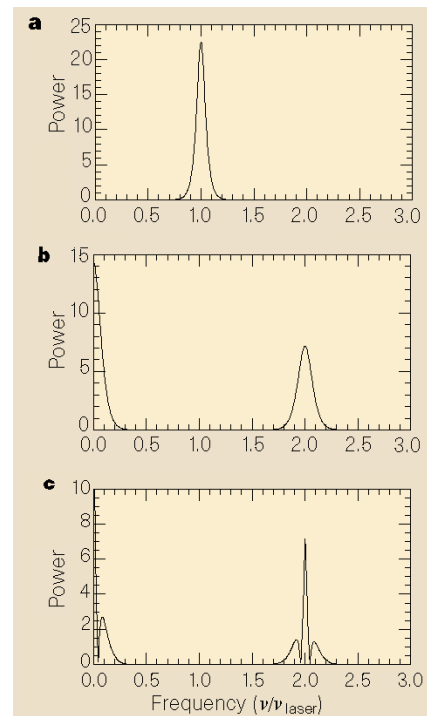


Figure 2 Spectral power distributions. a, The spectrum of the pulse in Fig. 1. b, The two-photon spectrum of the same pulse. c, The two-photon spectrum of a similar pulse, whose spectral components with frequency greater than the mean have been reversed in sign. The two-photon nonlinear absorption rate is sharpened.

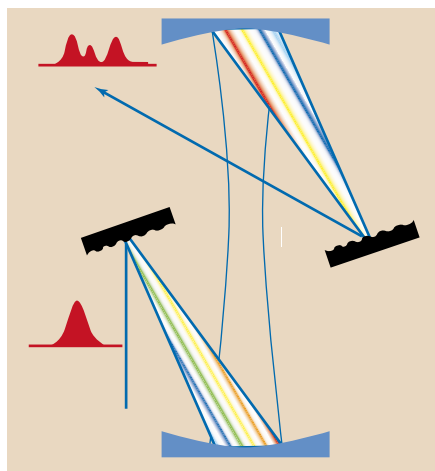


Figure 3 A pulse shaper, capable of imposing selective phase changes on a laser pulse.

Now if there is no resonance at the principal frequency, but one at twice that frequency, then absorption only occurs for pairs of photons. The atom no longer responds resonantly to the applied electric field, but rather to the square of the field  $E^2(t) = A^2(t)\cos^2(2\pi\nu t)$ , (the probability of a single photon's presence is proportional to the field, so the probability of two being present at once is proportional to the square). That has a completely different frequency spectrum (Fig. 2b), one peak of which happens to be at twice the laser frequency—that is at the position of the nonlinear resonance for two-photon absorption.

If one now adjusts the various phases of the different colours (or frequencies) in the initial spectrum, but leaves all of their amplitudes just the same, then the spectral energy density of the light field  $E(t)$  stays the same, but the spectral density of the nonlinear field shown in Fig. 2b can change. For example, let's reverse the sign of the upper half of the spectrum in Fig. 2a; in other words, transform the spectral components above the laser frequency from cosine waves to minus cosine waves. The power spectrum of  $E^2(t)$  now looks like Fig. 2c. By manipulating phases, the nonlinear spectral density can change so much that any particular resonant frequency in the spectrum can be made to vanish, and absorption at that frequency will vanish as well.

To achieve this degree of control, the Weizmann group made use of a new tool in optics called a pulse shaper<sup>2</sup> (Fig. 3). It is essentially two spectrometers arranged back-to-back. The first spectrometer disperses the light into its various colours, which are displayed along the exit plane of the device. At this exit plane are a series of filters side-by-side, which can attenuate or delay separate segments of the pulse spectrum. Meshulach and Silberberg's filters were parts of a programmable liquid crystal display, not very different from the one used in laptop computers, which could adjust the

phase delay of each frequency band. The second spectrometer then puts the separated colours back together again, and the result is a pulse with the same colours as before, but arranged with different relative phases and/or amplitudes.

To the original pulse the researchers added a frequency modulation that changes the phase, not the amplitude, of any frequency; but its effect on the nonlinear absorption spectrum was dramatic. As they describe in their paper, the two-photon resonance could even be made to vanish.

This dependence of quantum processes on the coherence of the driving radiation has already been used to control molecular dissociation and ionization pathways<sup>3</sup>, current in semiconductors<sup>4</sup>, and the direction of photoemission<sup>5</sup>. Ultrafast pulse shapers, which make it possible to manufacture very complicated phase distributions, have been used in time-domain coherent control experiments, where the control is achieved by manipulating the internal motion of a molecule, to guide it to a particular set of final states<sup>6</sup>. Pulse shapers have also been used to shape quan-

tum wave packets in atoms<sup>7</sup>. The new use of computer-controlled programmable pulse shapers in nonlinear interference experiments adds further capabilities to the field of coherent control. This could facilitate different chemical reactions in applications where laser chemistry is important, such as photolithography. A shaped nonlinear spectrum could also aid or inhibit atomic motion in molecules or in solids, where the energies of the two photons subtract rather than add (Raman interference). □

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#### Developmental neurobiology

## First class way to develop a brain

Jonathan Howard and Ian Thompson

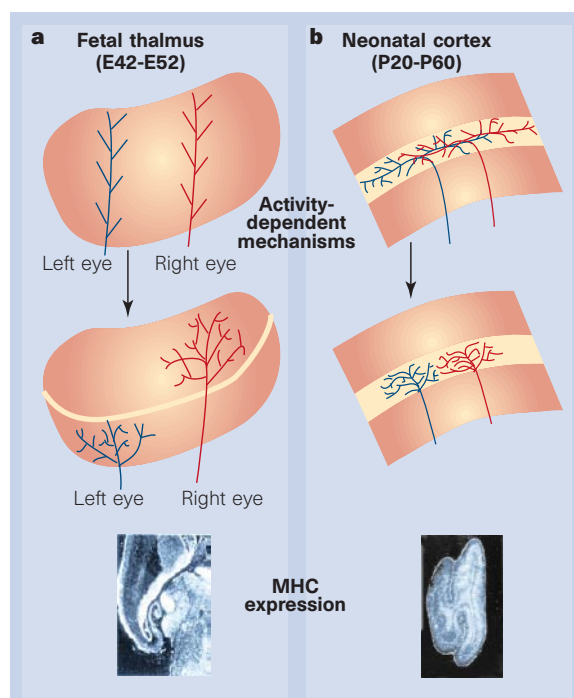
The major histocompatibility complex (MHC) is unambiguously part of the immune system. Generally speaking the immune system operates outside the blood–brain barrier, and this has been correlated with the belief that MHC glycoproteins are not expressed in the normal central nervous system. But this comforting correlation is now challenged by the radical observations of Carla Shatz and colleagues, published in *Neuron*<sup>1</sup>. They not only demonstrate, for the first time, considerable amounts of surface-expressed MHC glycoproteins in the optic system, but they also show that these proteins may be involved there in synaptic remodelling during development.

Shatz and co-workers were searching for genes that are differentially regulated in a region of embryonic cat brain called the lateral geniculate nucleus (LGN). They looked specifically between days 42 and 52 of gestation, when retinal input from the two eyes segregates into eye-specific zones. This activity-dependent repatterning of synaptic connectivity is blocked by tetrodotoxin, so the authors compared transcription in the LGN in the presence and absence of this toxin. They found just one messenger RNA that was differentially expressed, and this turned out, most surprisingly, to be the product of a known feline class I MHC gene.

Class I molecules usually present peptides derived from intracellular pathogens (such as viruses and bacteria) to the immune

system. In the brain, most normal, resting cells have vanishingly low levels of MHC class I (ref. 2). Now, however, Shatz and colleagues show, through elegant *in situ* hybridizations using feline class I MHC probes, that spatio-temporal expression of both the mRNA and protein correlates closely with synaptic remodelling in the development of binocular vision.

Shatz *et al.* demonstrate that expression of class I MHC within the LGN is highest in the late fetal and early neonatal stages of development. This period is well after axons from the retina first reach the LGN, but is when their terminal processes segregate into eye-specific zones and their morphology is fine-tuned (Fig. 1a, overleaf). Segregation and fine-tuning depend on neuronal activity<sup>3,4</sup>, but it is the pattern rather than the level of activity that is important. In the late fetal period, populations of retinal ganglion cells spontaneously fire short bursts of action potentials and then remain silent<sup>5</sup>. Although neurons in the same place tend to fire at the same time within each retina, there is no correlation between firing patterns in the two eyes. Tetrodotoxin blocks the eventual segregation of inputs from the two eyes in the brain, so this simultaneous firing seems to be a kind of link among the ganglion cells of each eye. Thus, if 'neurons that fire together, wire together', the pattern of spontaneous activity will ensure segregation of input from the two eyes in the LGN.



**Figure 1 Segregation of axonal terminals into eye-specific zones coincides with high expression of the major histocompatibility complex (MHC) in the target.** a, Axons from ganglion cells in the retina terminate in a thalamic nucleus, the lateral geniculate nucleus (LGN). In fetal cats, the axon terminals from the two eyes initially overlap and then segregate to terminate in distinct layers of the LGN. This process requires neuronal activity and coincides with high levels of MHC expression in the LGN. b, The axons of geniculate neurons terminate in the central layer of visual cortex. Axon terminals connected to the two eyes start off overlapping and then remodel to form eye-specific patches in the cortex.

Information from the two eyes is relayed from the LGN to the visual cortex where again the connections literally overlap before segregating into eye-specific zones. Much later in development, after birth, differences in the pattern of visually driven rather than spontaneous activity in the two eyes drives segregation of axon terminals from the LGN. Segregation occurs 20 to 60 days after birth and, indeed, Shatz and colleagues find that class I MHC expression is also selectively increased in the visual cortex at this time (Fig. 1b). So for both the LGN and the visual cortex, high levels of class I MHC are associated with remodelling of axon terminals.

But how is the MHC acting? Is its expression required on the remodelling axon terminals themselves, or is it required on the target neurons? Shatz and co-workers show that injections of tetrodotoxin into one eye,

38–48 days after birth, reduce MHC expression in the LGN neurons postsynaptic to that eye. But these injections also cause the terminals of the LGN neurons in the cortex to be rapidly reshaped<sup>6</sup>. So, if class I MHC is necessary for remodelling of axon terminals, levels in the input neurons cannot be important. Consequently, the prediction is that levels of MHC in the cortex can be sustained by functional inputs from just one eye. But blocking activity in both eyes, which freezes the segregation of neurons in the LGN<sup>7</sup>, should dramatically reduce expression of MHC in the cortex.

If class I MHC expression links patterned neuronal activity and axonal remodelling then an MHC receptor of some kind should also be involved, perhaps on the axonal terminals (Fig. 2). Sensibly, Shatz and colleagues looked, not for the classical T-cell

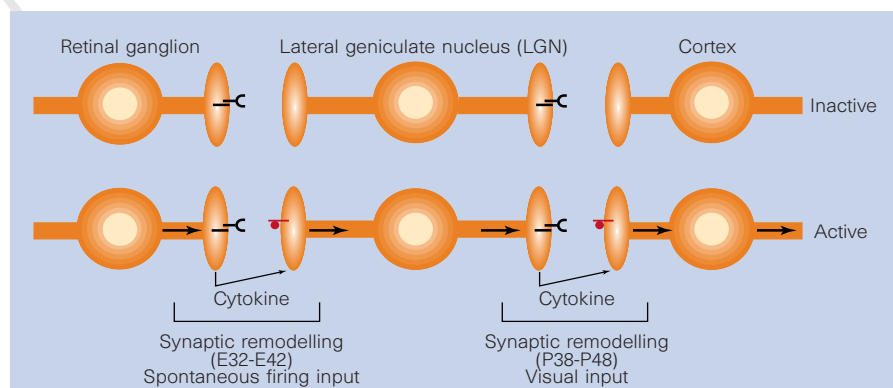
receptor (TCR) itself, but for a component of its cytosolic signal-transduction module, the TCR- $\zeta$  chain. They found TCR- $\zeta$  in the LGN and other thalamic nuclei. However, unlike class I MHC, the levels of mRNA were not regulated by neuronal activity.

Should the TCR- $\zeta$  chain be part of an endogenous receptor for class I recognition in the central nervous system, what is the receptor likely to be? Perhaps immunoglobulin-domain-based receptors similar to the activating receptors associated with natural killer cells<sup>8</sup> may be involved. Some of these receptors are probably stimulated by class I MHC molecules, without regard to their specific peptide content, and are thus well suited for a physiological role.

Expression of class I MHC can be induced in neurons and glial cells by inflammatory cytokines (such as the interferons and tumour-necrosis factor- $\alpha$ ) during inflammation and infection<sup>9</sup>. Does an equivalent cytokine induce class I MHC in the optic system during synaptic remodelling? Perhaps one of the neurotrophins, or a cytokine such as leukaemia inhibitory factor (LIF), may be involved (reviewed in ref. 10). LIF is expressed in the nervous system and is upregulated after injury or seizure. It has pro-inflammatory properties: there is diminished infiltration of inflammatory cells after brain injury in mice that lack the LIF gene, relative to wild-type animals (P. H. Patterson, personal communication). In both neural injury and seizure, expression of class I MHC is also induced<sup>11</sup>, and the effects of LIF, neurotrophins<sup>10</sup> and inflammatory cytokines<sup>12</sup> on neurons are known to be regulated by electrical activity.

It must have been exciting to find a differentially expressed gene in the remodelling LGN. What a surprise, then, to discover that not only the gene itself, but almost every conceivable gene involved in its expression and recognition, had long since been knocked out by others, and that the appropriate mice for all the obvious and not so obvious experiments were easily available. Nobody has yet reported a neural development phenotype in mice that lack class I MHC, nor in mice deficient in the transduction system at the receptor side. But the remarkable data of Shatz and colleagues mean that such animals should be re-examined.

Finally, the immune system may be invading the brain in more ways than one. The presence of class I MHC antigens in subsets of neurons suggests that these neurons may be selectively sensitive to immune pathologies. Shatz and co-workers find little MHC expression in another visual relay, the superior colliculus, and there are hints of differential MHC expression within the normal LGN. Given these differences in the cat, could the deficits in the magnocellular layers of the LGN in human dyslexics<sup>13</sup> be precipitated by differential expression of class I MHC



**Figure 2 Speculative model for the function of class I MHC in synaptic remodelling, based on the results of Shatz and colleagues<sup>1</sup>.** A hypothetical receptor (blue) containing the T-cell receptor  $\zeta$  chain is expressed on the axonal side of the synapse. Electrical activity stimulates the expression of class I MHC (red) on the dendritic face of the synapse, perhaps through induced local secretion from glial cells of a neurotrophin or a cytokine such as leukaemia inhibitory factor (as suggested by discussion with Paul H. Patterson). Recognition of MHC causes activation through the receptor containing the  $\zeta$ -chain, and reinforcement of the synapse through a tyrosine-kinase-mediated activation cascade.



antigens? Strikingly, there is evidence that dyslexia is genetically linked to the MHC region<sup>14</sup>. □

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## Photosynthesis

# A crystal clear view

Andreas Engel

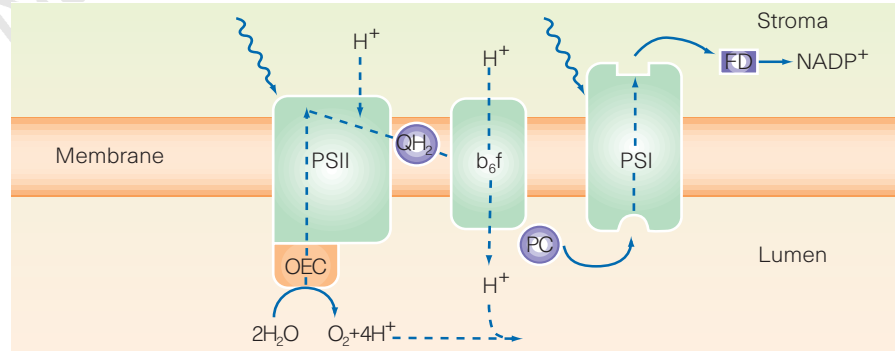
Photosynthetic bacteria, algae and plants have developed efficient systems to capture and convert sunlight into chemical energy. They produce about ten billion tons of carbohydrates annually, equivalent to eight times mankind's energy consumption in 1990. The first step in this reaction — light-driven electron transfer across the photosynthetic membrane — is executed by pigment–protein complexes known as the type-I and type-II reaction centres. The quantum yield (nearly 100%) and efficiency (about 40%) of this process, whereby photons are converted into electrochemical energy, are remarkable.

The structures of bacterial type-II and the cyanobacterial type-I reaction centres have already been solved by X-ray crystallography<sup>1–3</sup>. Now, on page 283 of this issue, Rhee et al.<sup>4</sup> describe their electron crystallographic analysis, which gives a first insight into the three-dimensional structure of the plant photosystem II (PSII) complex. This remarkable study also provides solid evi-

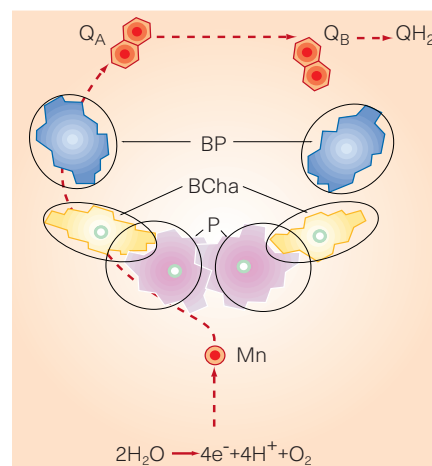
dence for a common evolutionary origin of the different types of reaction centre.

Whereas the type-I reaction centres found in green sulphur bacteria use iron–sulphur centres as terminal acceptors of the electron cascade, the type-II reaction centres of purple photosynthetic bacteria deliver the photoelectron to terminal quinones. In aerobic cyanobacteria, algae and, most importantly, plants, a more complex system has evolved (Fig. 1). This system can not only convert light into electrochemical energy, but it can also split water to produce oxygen by virtue of the oxidizing power of the activated PSII reaction centre.

The atomic structure of the reaction centre from the purple bacterium *Rhodospseudomonas viridis* was determined<sup>1</sup> in 1985, revealing the components of the electron pathway. This spurred spectroscopic experiments, mutagenetic analyses and computer simulations to unveil the main charge-separation mechanism. Photoelectrons are thought to move from the 'special



**Figure 1** The cascade of type-I and type-II light-driven electron pumps that have been tuned over billions of years to work together efficiently. Electrons extracted from water molecules by the oxygen-evolving centre (OEC) flow to quinones, which detach from the plant photosystem II (PSII) complex after being protonated. Reduced quinones (plastoquinol, QH<sub>2</sub>) diffuse in the bilayer to cytochrome b<sub>6</sub>f, a redox protein in the membrane that links PSII and photosystem I (PSI). Cytochrome b<sub>6</sub>f extracts electrons from plastoquinols to reduce plastocyanin (PC), the water-soluble electron donor for the PSI reaction centre. Protons are released into the lumen as plastoquinol is oxidized. PSI, the second light-driven electron pump in the system, transfers electrons to its terminal iron–sulphur centre. This, in turn, reduces ferredoxin (FD), which is the soluble electron carrier that reduces nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>).



**Figure 2** Active constituents of the electron pathway in the *Rhodospseudomonas viridis* reaction centre. The components are arranged symmetrically and include the special pair of chlorophylls (P), two bacteriochlorophylls (BCha), two bacteriopheophytin molecules (BP), and the terminal quinones (Q<sub>A</sub> and Q<sub>B</sub>). This geometry is similar in the PSII reaction centre (black contours), where the manganese (Mn) cluster accumulates the four oxidizing equivalents required to produce one molecule of dioxygen.

pair' of chlorophylls (P in Fig. 2) down a redox potential gradient through a series of acceptors (one bacterial chlorophyll and a bacterial pheophytin) to the quinones, Q<sub>A</sub> and Q<sub>B</sub>, tunnelling through intervening spaces of non-conducting organic matter<sup>5</sup>. Because tunnelling rates depend on the gap between successive acceptors (about an order of magnitude per Å), the protein structure that holds the acceptors must be rigid. This may explain why most of the membrane proteins that have been crystallized so far are involved in electron-transport processes.

The structure of the plant photosystem I (PSI) reaction centre at 4 Å resolution was described last year<sup>3</sup>, showing the arrangement of the 'special pair' and consecutive chlorophylls, as well as the three iron–sulphur centres that form the electron pathway. However, the PSII reaction centre has not yet delivered three-dimensional crystals that are suitable for high-resolution X-ray studies. So, Rhee et al.<sup>4</sup> have produced two-dimensional crystals from a PSII sub-core complex, comprising the D1, D2 and CP47 proteins that anchor the prosthetic groups. These authors then determined its three-dimensional structure to 8 Å resolution by electron crystallography.

By carefully comparing this structure with the atomic structure of the bacterial type-II reaction centre, Rhee and colleagues find that the PSII D1 and D2 subunits are similar to the bacterial L and M subunits, respectively, as expected from sequence homology. More surprising is the amazing match between the six helices that the

authors ascribe to CP47 and the first six helices of the PsaA/B subunits in PSI, because only a weak sequence homology has been noted between the PsaA/B helices and those predicted for CP47 and CP43 of PSII. This match is strong evidence that PsaA/B, which are essential subunits in the PSI reaction centre, have evolved from a gene fusion of a CP43/47-like protein with a five-helix prototype of a bacterial reaction centre.

Despite the limited resolution, the tetrapyrrole pigments can be detected in the PSII map by comparison with the bacterial reaction centre (Fig. 2). Two elongated densities that partially overlap with the tetrapyrroles of the bacterial special pair probably represent the two chlorophylls of the primary electron donor, P680. Interestingly, these densities are slightly further apart than in the bacterial reaction centre — 11 Å instead of 7.6 Å — consistent with spectroscopic analyses<sup>6</sup>. Further densities in the D1/D2 complexes could tentatively be assigned to the pheophytins, and densities corresponding to chlorophylls have been

identified in the CP47 map.

Rhee and colleagues' three-dimensional map of the sub-core of the PSII reaction centre not only gives us a framework on which to model this enzyme from the related structural elements of the bacterial and PSI reaction centres, but also shows how the pigment-protein complexes that convert sunlight into chemical energy have evolved. To understand the structure and function of these amazing enzymes is increasingly important in view of the urgent need to find a regenerative replacement for fossil fuels. □

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## Atmospheric chemistry

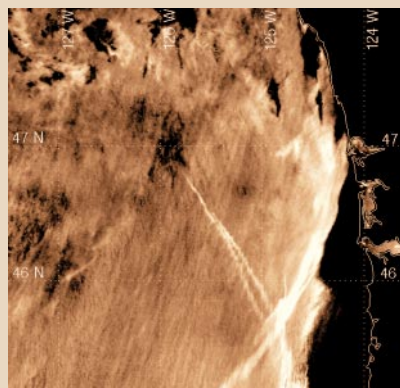
### Shipping forecast is partly cloudy

Aircraft contrails are a familiar feature of modern life, but ships too can leave traces of their passage in the sky. As this satellite image shows (R. J. Ferek *et al.*, in *J. Geophys. Res.* **103**, 23199–23206; 1998), diesel-powered vessels leave behind 'ship tracks' — narrow lines of perturbed regions in the marine atmosphere resembling bright bands of cloud.

Ship tracks were first reported more than 30 years ago. They are caused by the interaction of particles emitted from ships with ambient air, which results in a higher cloud droplet concentration and lower average droplet size. The consequence is increased cloud radiance at 3.7 µm, as detected by the advanced very-high-resolution radiometers (AVHRR) on satellites of the US National Oceanic and Atmospheric Administration.

On 26 August 1992, the region off the coast of Washington state was covered by a uniform layer of low-lying stratus clouds — the perfect weather to look for ship tracks on satellite images, and direct an aircraft to collect microphysical and chemical data in and around them. The two tracks spotted by Ferek *et al.*, seen in this picture as a narrow, inverted V, are caused by plumes from the *Forest Wave* and *Al Alamira* on their way northwest across the North Pacific Ocean.

The data collected during repeated aircraft crossings of the tracks, at about 210 m above the sea surface, show the expected strong perturbation of microphysical cloud properties: the initially very high total concentration of particles in the tracks



decreases over time, while the cloud droplets (which form in the presence of activated particles known as cloud-condensation nuclei) are about six times more abundant and half as large as those in the surrounding unperturbed clouds. Surprisingly, the higher droplet concentrations persist for a long time.

The radiative properties of clouds influence the global radiation budget, and depend sensitively on droplet size and concentration. Anthropogenic pollutants such as sulphur dioxide, volatile organic compounds and particulates can initiate the formation of cloud-condensation nuclei, and thereby perturb these radiative properties. Ship tracks seem an ideal setting for tackling the challenge of quantifying the correlation between emissions and cloud perturbations, and so may help to improve understanding of Earth's radiation budget and the factors affecting it.

Magdalena Helmer

## Daedalus

### Fossil knowledge

What we know of ancient societies comes largely from written records — paper and vellum documents and baked clay tablets. Clay tablets are by far the most enduring. Organic documents have mainly survived by preservation and repeated copying over the centuries. What does this imply for our own torrent of data?

The obvious conclusion is that it is all doomed. Magnetic and optical tapes and disks will delaminate and embrittle; paper and film will bleach and crumble. Worse, respect for the past is now dead. Modern short-term businesses, election-driven governments and fashion-crazed media have no use for archives; a future Dark Age might not bother with copying at all. Only a few 'clay tablets', in the form of commemorative statuary and crockery, will survive to puzzle future historians.

But Daedalus is more optimistic. He points out that fossilization can preserve animal remains over millions of years. Their organic content is replaced by stable mineral, and with amazing fidelity. So DREADCO chemists are trying to fossilize books, film, CDs, magnetic tapes and disks and so on. They are immersing them in pressurized carbonated or silicated water, and studying the rate and fidelity with which their organic content is replaced by the mineral. Daedalus argues that their information content will be unchanged. It is either held as a spatial pattern (as in CDs and gramophone records) or as a stable mineral itself: metallated carbon ink in printed paper, silver grains in monochrome film, magnetic oxide particles in tape and disks. Fossilized information media would faithfully carry their message down the millennia.

Reading them, however, will be tricky. A carbonate disk would be too brittle to be played; a silica reel of tape or film could not be unwound. The DREADCO team are studying their data-rich fossils by X-ray and magnetic-resonance tomography, trying to read them directly as three-dimensional solid objects. They hope to define the optimum procedure for recovering data from the mineralized remains of our time.

But how to inform future historians of this crucial work? Daedalus plans to encode his findings on the glass fibre read-only memory, or FROM, he devised last week. Fully mineral already, it is the only truly future-proof data format. FROMs will be the key by which our descendants will unlock the copious fossilized glories and absurdities of our civilization.

David Jones



# Merian's metamorphoses

**Nurtured from an early age in the art of still-life painting and naturalistic illustration, the courageous seventeenth-century artist Maria Sibylla Merian allied her vision and her skills to convey the complex life-cycles of insects.**

## Martin Kemp

For a 52-year-old painter who specialized in illustrating plants and insects to make a self-financed voyage to Surinam in 1699 to document the metamorphosis of exotic butterflies and moths is remarkable enough. For a woman, accompanied only by her 21-year-old daughter, it represents one of the most heroic acts in the history of the natural sciences. Maria Sibylla Merian of Frankfurt was an extraordinary person. She was also responsible for forging a new vision of how the life-cycles of insects could be brought before our eyes.

Her 'ecological' presentation, which she pioneered in *The Wondrous Transformation of Caterpillars and their Remarkable Diet of Flowers* in 1679 and 1683, found its finest expression in her *Metamorphosis of the Insects of Surinam* in 1714. The eggs, larvae, chrysalises and mature insects are portrayed in living communion with the plant on which their "worms" feed.

In her depiction of the moth *Arsenura armida* on the "Palisade tree" — so called because it provides the thick poles from which "the huts in America are built" — the stages in the life-cycle are interwoven with the plant in a living tapestry. As she explains: "Each year this kind of caterpillar comes three times to this tree; it is yellow with black stripes and decorated with six black spines. When they have reached a third of their final size they shed their skin and become orange-yellow with black spots on their limbs .... Several days later they shed their skin once more; on 14 April 1700 they turned into chrysalises; on 12 June moths like those emerged. The lower and smaller is the male; the larger and the upper one the female."

**Her aim was not systematic classification, anatomical description or priority in academic disputes, but to conduct us on a visual journey through the wonders of transformation.**

How was this vision conceived, when it is so different from the taxonomic 'portraits' of organisms found in the standard books? It can be seen as an extension of the kind of art in which she was steeped. Still-life painting and naturalistic illustration were in her blood.

She was the daughter of Matthias Merian the Elder, an engraver, illustrator and publisher, and her stepfather was the still-life painter Jacob Marrell. She married the painter Johann Andreas Graff. The kinds of still lifes that she learnt to paint with much skill were replete with descriptive detail and



Maria Sibylla Merian's "Palisade tree" (*Erythrina fusca*) with the moth *Arsenura armida*, from *De Metamorphosis insectorum Surinamensium*, 1714.

composed with decorative intent, but they also frequently embodied implicit narratives that remind us of the passing glories of earthly beauty. Maggots create decay at the heart of luscious fruit; leaves are holed by caterpillars; and gorgeous butterflies flutter through their transitory existence.

Should we wonder whether this 'vanitas' motif was relevant to natural history, we need only cite the title of the publication devoted to the mayfly by the microscopist and entomologist Jan Swammerdam, *A Figure of Man's Miserable Life* (1675). Merian is not so explicit, and the beauty of her observations and vivid depictions are sufficient unto themselves. But, as a pious believer, she knew that she was

working for an audience who would place her world of transformation in the proper theological context. She would have known that description, decoration and divine message were inseparably conjoined within a temporal framework.

As she had written in the preface to her *Transformations*, "I moved to present God's miracles .... Thus do not seek to praise and honour me for this work, but rather God, glorifying him as the creator of even the smallest and most insignificant of these worms." □

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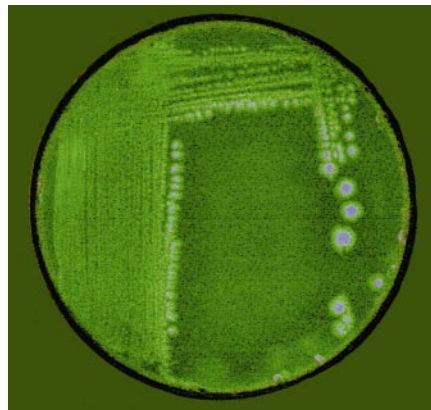


# *Clostridium* used in mediaeval dyeing

Before chemical methods of reduction were introduced in the past century, dyeing using the pigment indigo required a fermenting woad vat to reduce the insoluble indigo to a soluble form<sup>1,2</sup>. We have reproduced mediaeval techniques of woad preparation<sup>3</sup> and fermentation, and find that a thermophilic *Clostridium* bacterium was responsible for the reduction of indigo.

Leaves from the woad plant (*Isatis tinctoria*) were prepared for the woad vat according to the mediaeval practice<sup>1</sup> described<sup>3</sup>. Couched woad<sup>1,2</sup> provided the substrate for setting up the woad vat, which has been successfully used to dye wool and cotton. Spontaneous fermentation in the woad vat resulted in the evolution of gases (identified as CO<sub>2</sub> and H<sub>2</sub>), acidification of the medium (neutralized by addition of alkali to pH 9), and solubilization of the indigo, causing the vat to turn greenish brown with a surface layer of oxidized blue indigo. The redox potential of the fermenting woad was –560 to –595 mV relative to the standard calomel electrode. The woad vat evolved a noxious odour that was notorious even in mediaeval times<sup>1</sup>. Analysis by gas chromatography mass spectrometry of the organic headspace volatiles showed they were predominantly dimethyl sulphide (45%), dimethyldisulphide (24%) and methanethiol (18%) (C. Duckham and J. Ames, unpublished data), which are characteristic volatile products of the anaerobic breakdown of cruciferous plant material<sup>4</sup> as well as of microbial metabolism<sup>5</sup>.

We enriched five isolates of indigo-reducing Gram-positive anaerobic bacteria from the fermenting woad vat by using indigo-supplemented, reinforced clostridial agar (pH 9) incubated at 47 °C. Reduction of indigo was indicated by the medium turning colourless under anaerobic conditions, and becoming blue on exposure to air (Fig. 1). All of the isolates were rod-shaped,



**Figure 1** Indigo reduction by *Clostridium* isolated from a woad vat. The plates were initially brown because, even though they contained indigo, the dye was not divided finely enough to colour the agar blue. *Clostridium* reduced the indigo anaerobically to its colourless form, then, when exposed to air, the indigo was reoxidized and the agar turned blue.

produced oval terminal endospores, and were negative for the enzyme catalase. Phylogenetic analysis and 16S ribosomal RNA gene sequence analysis<sup>6</sup> of a representative strain (Wv6) showed that the unknown bacterium was a member of rRNA cluster I *Clostridium*, related to *C. carnis*. *Clostridium* strain Wv6 is a phylogenetically distinct species for which the name *C. isatidis* will be formally proposed elsewhere<sup>7</sup>.

We conclude that it was the reduction of indigo by *Clostridium* that enabled indigo to dissolve and so allowed its use as a dye for textiles in mediaeval times. This finding constitutes another important biotechnological application of anaerobic microbes<sup>8</sup>.

The mediaeval vat operators were aware of several requirements for the system to work: they had to keep the vat alkaline, adding wood ash<sup>1,2</sup> to counter acid produced by fermentation; they provided the *Clostridium* with polysaccharide substrate,

adding bran to maintain the fermentation for several months<sup>1,2</sup>; and they had to maintain the temperature at around 50 °C (refs 1,2) as the *Clostridium* is thermophilic. The *Clostridium* also reduced indigo when inoculated as a pure culture in reinforced clostridial medium (pH 9), its growth being accompanied by the production of acetic, formic and lactic acids.

In current industrial practice, the dyeing of cotton yarn for denim production consumes about 2 kg of sodium dithionite for every 3 kg of indigo reduced<sup>9</sup>. Disposal of the by-products of dithionite oxidation is an environmental burden, which could be eliminated by replacing modern chemical methods of indigo reduction with one based on the fermentative biochemistry of indigo-reducing *Clostridium*.

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## Scale of mast-seeding and tree-ring growth

The synchronous production of large seed crops by a population of plants<sup>1,2</sup>, known as mast-seeding, and synchronous tree-ring growth<sup>3,4</sup> within sites are well known phenomena among trees in the temperate zone. But information about the geographic or taxonomic extent of such synchronous growth or reproduction, or about the geographic extent of switching between them, is sparse. We have detected synchrony in growth and reproduction, both within and

among genera of Northern Hemisphere boreal trees, across geographical areas almost the size of a continent. Furthermore, we found a significant negative correlation between seed production and tree-ring growth at sites up to 1,000 kilometres apart, implying that there are trade-offs between them. This discovery suggests that mast-seeding is an evolved strategy that occurs on a geographic scale far larger than previously suspected<sup>5</sup>.

Annual seed or cone production values were obtained from the literature and standardized so that data acquired using different scales and methodologies could be combined. We used 298 data sets from

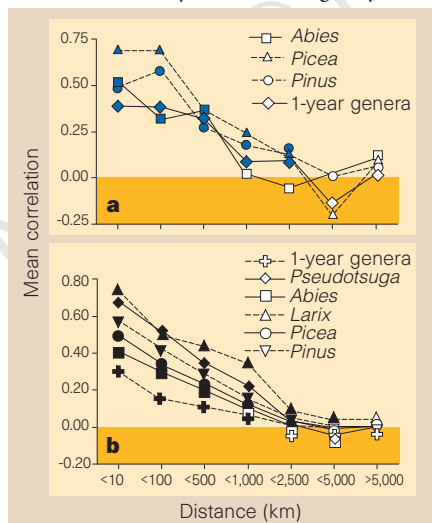
seven genera of boreal coniferous trees (*Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, *Thuja* and *Tsuga*) containing a minimum of four years of data (mean, 12.4 years). As a proxy of vegetative growth, we used dendrochronologies obtained from the International Tree-Ring Data Bank (version 2.2), a repository of more than 1,200 site-standardized chronologies from around the world.

Both data sets consisted of annual site means. Analyses included within-genus comparisons of sites measuring congeners and among-genera comparisons performed by pairing different genera. This latter analysis included firs (*Abies*), larches (*Larix*),

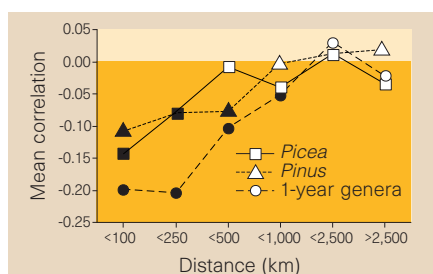
spruces (*Picea*), Douglas-firs (*Pseudotsuga*), cedars (*Thuja*) and hemlocks (*Tsuga*), but excluded pines (*Pinus*), which require more than a year to mature cones. Spatial autocorrelations and cross-correlations were calculated using a modified correlogram procedure<sup>6</sup>.

Our results reveal statistically significant spatial autocorrelation over large areas. Synchrony in the production of seeds and cones was detectable between sites from 500 km apart (*Abies*) up to 2,500 km apart (*Pinus*) (Fig. 1a). Synchrony in tree-ring growth was found between sites from 500 km apart (*Abies*) up to 5,000 km apart (*Larix*) (Fig. 1b). Assuming a circular area with a diameter corresponding to these distances, this indicates that detectable synchrony in cone production occurs over areas of  $0.2\text{--}4.9 \times 10^6 \text{ km}^2$ , and tree-ring growth patterns over areas of  $0.2\text{--}19.6 \times 10^6 \text{ km}^2$ . For comparison, the approximate land area of the North American continent is  $24.3 \times 10^6 \text{ km}^2$ , so reproduction and radial growth of conifers are statistically synchronous over subcontinent- to continent-wide areas.

Cone production and tree-ring growth patterns are both generally correlated with climatic factors, such as temperature or rainfall<sup>3,4,7</sup>. If the large-scale patterns of variable growth and reproduction are positively correlated with climate, they may involve only environmental tracking and require no further evolutionary explanation. Alternatively, mast-seeding may be an



**Figure 1** Correlations between synchronous activity of Northern Hemisphere coniferous trees depending on the geographical distance between sites. **a**, Mean annual seed or cone crop; **b**, mean annual tree-ring growth. Within-genus comparisons (*Abies*, *Larix*, *Picea*, *Pseudotsuga* and *Pinus*) include all sites measuring any species within the genus. The one-year genera category involves among-genera correlations using *Abies*, *Larix*, *Picea*, *Pseudotsuga*, *Thuja* and *Tsuga*, and excluded all within-genus comparisons. Filled symbols are significant at  $P < 0.05$  by randomization tests.



**Figure 2** Cross-correlation between mean annual seed or cone crops and mean annual tree-ring growth of Northern Hemisphere coniferous trees depending on the geographical distance between sites. Within- and between-genus comparisons are as for Fig. 1; only *Picea* and *Pinus* had sufficient data to be tested individually. Filled symbols are significant at  $P < 0.05$  by randomization tests.

evolved strategy, conferring fitness advantages by means of economies of scale involving seed predation, seed dispersal, or wind pollination<sup>8,9</sup>. The critical prediction, given these alternatives, is that there must be a trade-off between vegetative and reproductive growth on a population scale<sup>1</sup>.

We tested for such trade-offs by using cross-correlation analyses between the tree-ring and seed-production data sets. These yielded a negative relation between growth and reproduction for sites up to 250 km apart for spruces, 500 km apart for pines, and 1,000 km apart for one-year genera combined (Fig. 2). These inverse correlations between growth and reproduction, which are indicative of resource switching, are detectable within genera across areas of up to 196,000 km<sup>2</sup> and between genera across areas of up to 785,000 km<sup>2</sup>.

Our results show that mast-seeding involving switching of resources between growth and reproduction is characteristic of Northern Hemisphere conifers on a large geographic scale. Furthermore, in conifers, mast-seeding is detectable both between species and between genera, at least when restricted to genera requiring a single year to mature cone crops. They also show that patterns of seed production are sufficiently broad, both taxonomically and geographically, to affect the populations of seed predators over large areas. For example, our results support the hypothesis that the synchronized invasion of boreal seed-eating birds into southern latitudes could be the result of geographically widespread seed-crop failures<sup>10</sup>. Similarly, resident populations of birds and mammals that depend on conifer seeds for food are likely to be affected synchronously over large geographic areas by both bumper crops and widespread crop failures.

The many potential effects of such large-scale ecological phenomena on ecosystem function and biodiversity<sup>11</sup> remain to be investigated. Furthermore, because broad

climatic patterns almost certainly correlate with the large-scale synchrony, global climate change<sup>12,13</sup> may affect ecosystems in previously unanticipated ways.

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## Niche adaptation in ocean cyanobacteria

Small unicellular cyanobacteria (*Synechococcus* and *Prochlorococcus*) are the most abundant photosynthetic microorganisms in the world's oceans, yet we know little of the genetic structure of these populations. Here we show that distinct clades of *Prochlorococcus*, and possibly of *Synechococcus*, are adapted to their specific surface and deep oceanic niches.

Genetic and physiological studies of cultivated *Prochlorococcus*<sup>1–5</sup>, together with the discovery that organisms living at different depths have differing cellular properties (such as their divinyl chlorophyll *b:a* ratios)<sup>6,7</sup>, have led to the hypothesis that the widespread abundance of *Prochlorococcus* can be explained by the ability of surface- and deep-adapted clades to use different niches. However, cultivated isolates and bulk properties such as pigments are inadequate as descriptions of natural cyanobacterial communities<sup>8</sup>.

We used the polymerase chain reaction (PCR) as previously described<sup>9</sup> to amplify a fragment of the cyanobacterial RNA polymerase C1 gene (*rpoC1*) from communities of bacteria inhabiting oligotrophic sites in the Pacific Ocean, off the coast of California. We then used sequences from the generated clone libraries to survey marine *Synechococcus* and *Prochlorococcus*

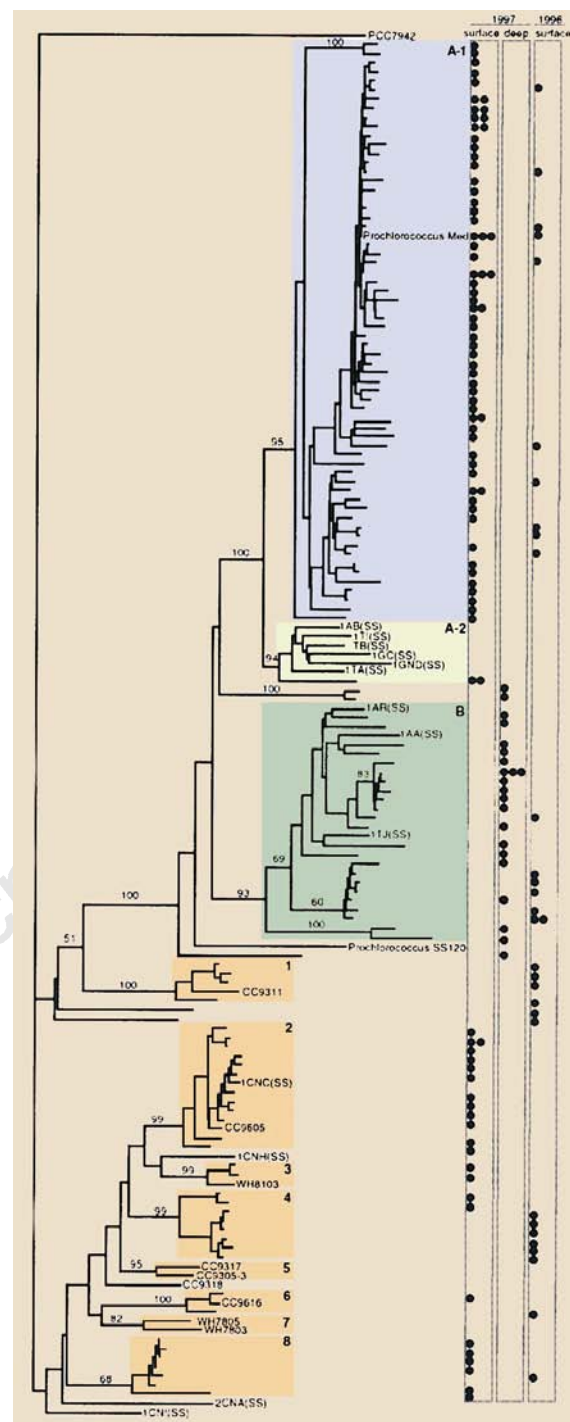
populations in surface and deep environments. We found a large number of distinct clades of these organisms, many of which are not represented by isolates in culture (Fig. 1).

We prepared three *rpoC1* libraries from samples collected in October 1997: two from station 77.100 (33° 23.2' N, 124° 19.6' W), consisting of one surface library from a depth of 30 m and one from the deep chlorophyll fluorescence maximum (DFM) at 110 m, and one from station 93.120 (29° 51.0' N, 123° 34.9' W), a surface library from a depth of 20 m. Fluorescence and temperature depth profiles of these two stations were nearly identical, and were typical of well stratified oligotrophic waters with a 110-m DFM and mixed layers at 19.5 °C. A fourth surface library, from a depth of 3 m, was prepared from samples taken at station 77.100 in October 1996, when the station exhibited a shallow DFM (50 m) and a cooler (17 °C) mixed layer.

*Prochlorococcus* sequences formed two major clades, which separated according to the depth of the clone's collection. In the two surface libraries from the typical oligotrophic stations, 66 of 68 *Prochlorococcus* sequences clustered within clade A-1 (Fig. 1). This, combined with three cloned A-1 sequences identical to that of *Prochlorococcus* MED, a Mediterranean Sea surface isolate (CCMP1378) that has been characterized as highly light-adapted<sup>4,5</sup>, led us to designate A-1 as 'surface types'. One *Prochlorococcus* sequence, represented by two clones, clustered with sequences previously obtained from a 60-m Sargasso Sea library<sup>9</sup> in group A-2 (Fig. 1). We suspect that these closely related surface clades could be distinguished by environmental factors other than light, particularly temperature.

In the library from 110 m, 19 of 22 sequences clustered within clade B (Fig. 1), so were designated as 'deep types'. Three deep-library clones formed well resolved *Prochlorococcus* lineages. Although chimaeric PCR products are always possible, we do not believe that these clones are chimaeras, and similarly deeply branching lineages have also been found by using 16S ribosomal RNA analysis of isolates. None of the surface types appeared in the deep library, and none of the deep types appeared in the two surface libraries from the typical oligotrophic stations.

The phytoplankton collected at station 77.100 in October 1996 show a different vertical structure, reflected in the different depth fluorescence profile at this site. The corresponding surface library (Fig. 1, last column) shows less stratified *Prochlorococcus* populations, with ten surface types (clade A-1) and seven deep types. The combination of deep-type *Prochlorococcus* and cooler surface temperatures may reflect



**Figure 1** The relationships between cyanobacteria from different environments. The dendrogram shows the results of neighbour-joining analysis with Jukes-Cantor-model distances in 100 bootstrap replicates using a sequence of 270 nucleotides at the carboxy-terminal region of the *rpoC1* gene fragment. Numbers on the branches show clades supported by more than 50% of bootstrap replicates. Clades discussed in the text are shown by shaded regions and bold lettering. Columns represent clone libraries; circles correspond horizontally to clones, and illustrate the distributions of sequences in different libraries. SS, Sargasso Sea clones; WH, Woods Hole strain; CC, California Current strain; PCC, Paris Culture Collection strain.

mixing, a shorter stratification period, or increased coastal influence.

*Synechococcus* sequences were detected in each surface library and can be divided into eight or more clades, indicating that genetic diversity in this group is more complex (Fig. 1). By analogy to *Prochlorococcus*, *Synechococcus* clades 2 and 8 might represent surface-adapted organisms, as, with one exception, bacteria from these clades were detected only in the 1997 surface libraries from stratified stations 77.100 and 93.120. Clade 2 is apparently also present in the oligotrophic Sargasso Sea<sup>9</sup>. Preliminary results indicate that isolate CC9605 from clade 2 grows well with white light (100  $\mu\text{E m}^{-2} \text{s}^{-1}$ )

at 21 °C, but not with low (15  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) blue light at 19 °C, in comparison with other isolates from the California Current.

The minimum sequence identity between members within the clades in Fig. 1 ranged from 95% (*Synechococcus* group 4) to 80% (*Prochlorococcus* group B). *Prochlorococcus* isolates MED and SS120, characterized as surface- and deep-adapted, respectively, were 76% identical here and more than 98% identical by 16S ribosomal RNA gene sequences<sup>2</sup>. Members of *rpoC1*-defined clades are therefore unlikely to be distinguished as different species by current standards. The definition of the clades presented here by ecotypic characteristics



requires further investigation. However, the dominant contribution to primary productivity by *Prochlorococcus*<sup>10</sup> is probably due, in part, to its divergence into distinct clades adapted to surface- and deep-water environments.

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## Why people gesture when they speak

People use gestures when they talk, but is this behaviour learned from watching others move their hands when talking? Individuals who are blind from birth never see such gestures and so have no model for gesturing. But here we show that congenitally blind speakers gesture despite their lack of a visual model, even when they speak to a blind listener. Gestures therefore require neither a model nor an observant partner.

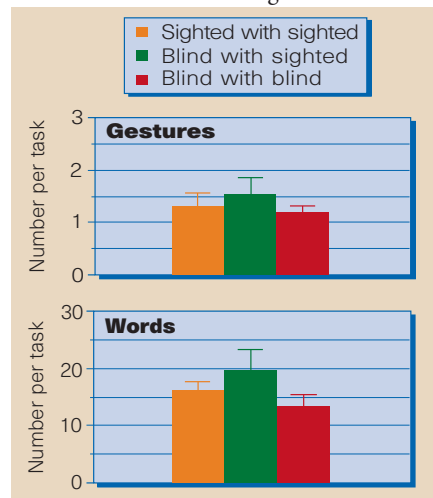
Gestures are produced by speakers from all cultural and linguistic backgrounds<sup>1–3</sup> and emerge in young children even before the development of language<sup>4,5</sup>. The spontaneous hand movements that accompany speech are not random but convey to listeners information<sup>6</sup> that can complement or even supplement the information relayed in speech<sup>7,8</sup>. Although a great deal is known about when and in what way speakers gesture, little is known about why they do it. We tested two possibilities that were not mutually exclusive.

The first possibility is that speakers gesture simply because they see others gesture, and learn from this model to move their hands as they talk. To test this idea, we studied spontaneous communication in 12 congenitally blind children and adolescents (4 males, 8 females) ranging in age from 9;1 (years; months) to 18;10 (mean, 12;10), and in a comparison group of 12 sighted children and adolescents (4 males, 8 females; ages 9;1–17;3, mean 11;11). The blind

participants had minimal light perception at best, and no other known cognitive, emotional or physical deficits. Sighted participants were matched to blind individuals on the basis of age, gender and ethnicity.

Participants were videotaped while responding spontaneously to a series of reasoning tasks known to elicit gesturing in sighted children<sup>9</sup>. Speech and gesture were transcribed and coded according to a system developed previously<sup>9</sup>. Hand movements were coded as gestures only when they did not involve direct manipulation or exploration of the objects, had a clearly identifiable beginning and end, and were temporally correlated with speech. Agreement between coders was 87–90% for identifying gestures and coding their form.

We found that all 12 blind speakers gestured as they spoke, at a rate not reliably different from the sighted group (Fig. 1), and conveyed the same information using the same range of gesture forms. For example, both blind and sighted speakers tilted a C-shaped hand in the air as though pouring liquid from a glass to indicate that a liquid had been transferred to a different container. Blind speakers do not seem to require experience of receiving gestures before they spontaneously produce gestures of their own. Sighted speakers of different languages are known to gesture at different rates<sup>3</sup>. Given our findings, we might expect that congenitally blind speakers of different languages would not mirror these cross-linguistic differences (unless, of course, there are cultural and linguistic influences



**Figure 1** Mean number of gestures and words produced per task by 12 sighted and 12 congenitally blind speakers interacting with a sighted experimenter, and 4 congenitally blind speakers interacting with a blind experimenter. There were no significant differences in either gesture (Mann-Whitney  $U = 65$ , n.s.) or word ( $U = 69$ , n.s.) production comparing blind with sighted speakers, or comparing blind speakers interacting with blind versus sighted experimenters ( $U = 21.5$  for gestures,  $U = 16$ , for words; both non-significant). Error bars show standard errors.

on gesturing that are transmitted at deeper levels than the eye).

The second possibility is that speakers gesture because they understand that gestures can convey useful information to the listener. To test this hypothesis, we examined whether speakers gestured even when talking to a listener known to be blind, and thus obviously unable to profit from information conveyed by gesture. We asked four additional children (1 male, 3 females; ages 5;0–8;6, mean 7;6), each blind from birth, to participate in the same reasoning task. These subjects were told that the experimenter herself was blind. Nevertheless, all of the blind speakers gestured, and did so at a rate not reliably different from that of sighted-with-sighted or sighted-with-blind pairings (Fig. 1). The 4 blind speakers interacting with a blind experimenter were younger than the 12 blind speakers interacting with a sighted experimenter. We therefore compared them with a subset of the 12 matched for level of performance on the tasks, and again found no differences in gesture or word production. Thus, blind speakers do not seem to gesture solely to convey information to the listener.

The relatively small number of speakers in the blind and sighted groups may have made it difficult to detect a difference in gesture usage. The important point, however, is that all 16 of the blind speakers produced gestures resembling those of the sighted speakers.

Our findings underscore the robustness of gesture in talk. Gesture does not depend on either a model or an observer, and thus appears to be integral to the speaking process itself. These findings leave open the possibility that the gestures that accompany speech may reflect<sup>7</sup>, or even facilitate<sup>10</sup>, the thinking that underlies speaking.

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# Careers down the tubes

## **The Making of the Chemist: The Social History of Chemistry, 1789–1914**

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£50, \$80

**Bernadette Bensaude-Vincent**

Although a history, this volume reveals a landscape so familiar to today's chemists that it could serve as a family album. It's all about university degrees, laboratory training, academic posts that combine teaching with research, scientific expertise, chemical societies, specialized journals with editorial boards and all-powerful referees, international conferences, and so on. When did this modern organization of scientific activity emerge? The answer is definitely in the nineteenth century. Chemistry, which was a popular and fashionable science at that time, has often been used as an example of the processes of professionalization, specialization and internationalization in science.

But where and how was a community of chemists brought into being? These questions are the focus of this volume, which is one of the results of a research programme sponsored by the European Science Foundation. One major outcome of this collaborative enterprise has been to show that there are no simple answers to such questions when they are addressed on a large scale.

From this rich collection of 18 contributions dealing with 15 different countries, it is clear that the standard model of professionalization fashioned after the British and German cases needs to be revised. For instance, the conventional view that chemistry emerged as an autonomous science by emancipation from medicine and pharmacy is not valid for such countries as Belgium and Denmark, where chemists and pharmacists formed a single community. Moreover, on a broad European scale, the close relationship established between academic chemists and the chemical industry in Germany appears to be an exception rather than the rule.

Each country has developed its own chemical culture. These cultures were shaped by national contexts; the bureaucratic centralization of countries like Russia and France contrasts with local initiatives prevalent in other countries. Chemical cultures were also heavily dependent on agricultural and industrial demands: dyes were developed for the booming British textile industry, chemists were needed in Belgium for the sugar industry, while in Sweden, chemistry was centred on blowpipe techniques developed in connection with mineralogy. The people who called themselves 'chemists' presented a variety of profiles and skills, and



A French nineteenth-century dyeing workshop, as depicted by René Joseph Gilbert.

ranged widely in their social status.

The organization of the volume into three sections suggests a concentric map of European chemistry. The centre of gravity is formed by 'the big three' — France, Britain and Germany. An inner ring of 'medium-developed countries' comprises Italy, Russia, Spain, Belgium, Ireland and Sweden, and the outer ring embraces peripheral countries such as Denmark and Norway, Portugal, Greece, Lithuania and Poland. The lazy reader who is satisfied with a glance at the table of contents would be totally misled by this apparent pattern, for the details of the contributions challenge the standard centre/periphery model. Certainly, there were leading countries as well as countries importing both foreign chemists and foreign models of education. But we must be aware that the very notion of leadership and national rival-

ries in science emerged during the period under scrutiny. During the second half of the nineteenth century, this trend was reinforced by regular world exhibitions and by international tensions and wars, especially the Franco-Prussian War of 1870–71.

In fact, as emphasized by the editors, there was no stable leadership, and the peripheries followed their own patterns of development and professionalization. More importantly, this book provides many examples suggesting that the centre/periphery model is by no means a measure of national development. One should be careful about using the number of publications as an indicator of 'maturity', or the creation of national scientific societies as a measure of professionalization. For instance, no French chemical society was founded before 1906, although France had a stronger



academic community in the nineteenth century than Russia, where a chemical society was founded as early as 1867. A major merit of this volume is its emphasis on the local circumstances, on the tensions between groups and the rivalries between local societies, which sometimes played a more significant role than national policy in the emergence of chemical societies and educational institutions.

Although it does not entirely escape the problems of heterogeneity inherent in collective volumes, this is much more than just a collection of national case-studies. It outlines the general trends, the main developments and the key actors in the process leading to the professional chemist, while providing a wealth of information that qualifies and diversifies the general scheme. In this respect, it is a model of collaborative publication, with genuine exchanges between the contributors and a thorough job of coordination by the two editors.

This volume should also prove useful to anyone exploring ways to improve communication between professional historians and practising scientists with a historical interest in their discipline. It has been repeatedly deplored that professional historians of chemistry do not write for chemical audiences, that they have been too concerned with local cases or with sociological questions to supply the demands of either the chemists who want a historical glimpse of their own achievements, status and activity, or the teachers eager to present chemistry from a historical perspective. This volume is well suited to fulfil both chemists' and historians' expectations. Chemists will find here the landmarks of nineteenth-century European chemistry. Professional

historians will enjoy the fine-grained analyses of various local contexts.

This strength was made possible, I think, because of the broad meaning given to 'social history'. It is not just understood as institutional history, but rather as a cross-section of intellectual, national and industrial history, as well as the history of education and economics. It is, therefore, regrettable that a book of this kind, suited to a wide international audience, is rather expensive. □

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## As the pigeon flies

### Spatial Representation in Animals

edited by Sue Healy

Oxford University Press: 1998. 187 pp.

£45, \$86 (hbk); £22.50, \$40 (pbk)

Nicola S. Clayton

As someone who struggles on a daily basis to remember where I left my car this morning, or even which direction on the freeway I should take, I marvel at the spatial memory and navigational capabilities of animals. What cues does a Siberian willow tit use to remember where it left its hidden food caches, given that it stored about half a million pine seeds in different sites throughout its territory several months earlier? How do monarch butterflies find the scarce, high mountain patches of the oyamel fir forest in central Mexico each autumn, more than 2,500 km away from the breeding grounds they just left? While the memory feats of a food-storing bird and the migratory capabilities of a butterfly may indeed be specialized examples, knowing how to get home is a problem most animals need to solve.

*Spatial Representation in Animals* brings together work on navigation and orientation in several different species, from honey bees to hamsters. It originated in a series of papers given at the 1995 winter meeting of the Association for the Study of Animal Behaviour, but, unlike many conference proceedings, the eight chapters in this book are easily accessible to advanced undergraduate and graduate students as well as to experts in the

field. Another appealing feature is that the authors come from a variety of disciplines — from animal behaviour and behavioural ecology to neurophysiology and cognitive psychology — and this creates an integrative feel.

The book begins with a discussion of how animals use landmarks: Ken Cheng and Marciel Spetch's chapter focuses on mechanisms of landmark-based spatial memory in pigeons and gerbils, while Jochen Zeil and Tom Collett's chapter provides a different perspective, focusing on how arthropods rely on landmarks to find their way around. The theme of landmark use is taken up again in a chapter by Etienne Save *et al.*, who discuss the concept of a 'cognitive map'. They emphasize the fact that rodents may rely on several different categories of cues when navigating, and that the relative importance of these cues may depend on how the experiment is set up. There is much debate about whether rats, and other animals for that matter, possess a 'cognitive map' and how useful the concept is anyway. Given such controversy, I was somewhat surprised to discover that the seminal paper by Andrew Bennett (*J. Exp. Biol.* **199**, 219–224; 1996) did not appear anywhere in the reference list, especially given his active participation in the conference.

Ariane Etienne *et al.* assess the importance of 'dead reckoning', or path integration, a mechanism of calculating distance and direction when travelling. Path integration allows the animal to make a straight-line return to its starting point without having to remember the landmarks that it has seen previously. As Etienne and co-authors point out, many animals, including humans, use path integration.

Another controversy involving the navigational mechanisms that animals use centres around the role of olfaction in pigeon homing behaviour, and it seemed particularly appropriate to find a chapter on this topic, by Verner Bingham, at a time when a consensus has finally been reached. The homing pigeon work is a wonderful example of the benefits of adopting an integrative approach to understanding the mechanisms of navigation at multiple levels. Salmon is another well-known model of homing, and the chapter by Victoria Braithwaite suggests that future work on the navigational and spatial memory capabilities of fish may be invaluable in extending our knowledge of animal spatial representations. This contrasts with the chapter by Peter Berthold, where the challenge is to summarize the vast body of literature on long-distance migration in birds.

A thorny area dear to my own heart concerns the role of the hippocampus in spatial representation, an issue that is addressed in the final chapter by David Sherry and Sue Healy. Evidence from both experimental

Canada geese migrating at night.



S. NIELSEN/BRUCE COLEMAN LTD



and comparative studies suggests that the hippocampus is involved in spatial learning, but whether or not this is a primary function of the hippocampus remains an open question. An increasingly common view is that the hippocampus is more generally involved in learning and remembering events that depend on complex, configural cues, with spatial tasks merely being good markers with which to detect hippocampal function.

*Spatial Representation in Animals* provides an illuminating and up-to-date introduction to our understanding of the ways in which animals find their way about. It is definitely worth a read. □

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## Chips off the old block

### Lost Civilisations of the Stone Age

by Richard Rudgley

Century: 1998. 307 pp. £17.99

John Robb

My father, an eager reader of archaeology, accuses me of refusing to believe anything fun about the past, and it is true that popular books almost always excite a curmudgeonly snobbery in the halls of academe. But it can be equally condescending not to take a popular treatment to task if it so deserves. Books written for non-archaeologists are also based upon theoretical ideas which, if they are important enough to write a book about, deserve critical thought.

In *Lost Civilisations of the Stone Age* Richard Rudgley makes a simple argument. Civilization did not arise fully formed with the first literate societies around five thousand years ago. Rather, virtually all of the things we associate with 'civilization' have precursors, if not direct ancestors, in the recent or distant prehistoric past, in the two million years of the Stone Ages. From this basis flows a series of archaeological vignettes, starting with the relatively recent prehistory of the Neolithic, leaping among the high points of the Upper Palaeolithic, and finishing among Middle and Lower Palaeolithic controversies about the very origins of humans and their tools and art.

Writing, surgery, drug use, monument building, detailed environmental knowledge, sophisticated artworks, technologies such as mining and smelting, language, musical instruments, tools fashioned with aesthetic sense as well as utilitarian function — all arose far earlier than either archaeologists have generally acknowledged or the public has imagined. The result is that we cannot consider our history as a simple story



This late Stone-Age passage tomb suggests the sophistication of so-called primitive societies.

of the 'rise' from savage roots to a sophisticated present. Our ancestors, even tens of thousands of years ago, commanded surprising knowledge and expert skills.

This panorama of prehistoric achievements makes wonderful reading. Rudgley, a fluent writer, is clearly fascinated with his material and his enthusiasm pervades the book, illuminating some little-considered corners of archaeological knowledge. To take just one example, the chapter on pyrotechnology rapidly reviews controversies over whether early hominids used fire, sketches the Upper Palaeolithic ancestry of lamps and fired-clay technology, and lucidly describes how many ancient people invented the technique of heating flint in fires to make it more supple and easily flaked. As Rudgley points out, with examples of ancient ingenuity abounding, there is no need to turn to ancient astronauts to explain civilization.

Rudgley almost always finds himself taking the minority view over controversies, and his conclusions are occasionally facile or follow discredited authorities. But throughout the book we find clear exposition, a refreshing straightforwardness about the complexity of the archaeological record, a willingness to explore many sides of an issue, and a zest for discovery that make it a page-turner.

Ultimately, however, Rudgley's basic argument forms something of a straitjacket for the exuberant archaeological material. The basic message — that prehistoric people were able to do all sorts of sophisticated things — makes waves only if one begins by assuming that all societies must either be 'civilized' or 'primitive', and that 'primitive' societies are necessarily backward in all regards. As Rudgley points out, this is

certainly not so. But, rather than trying to understand each prehistoric society on its own terms, Rudgley simply tries to show that prehistoric societies were also 'civilized'.

While understandable, this essentially Victorian approach to ethnology doesn't demolish the 'rise to civilization' storyline, as Rudgley intends it to; it merely redraws the line between who is in the civilization club and who isn't. By measuring all societies against the standard of 'civilization', Rudgley creates a pastiche of prehistoric societies, in which the differences between modern African tribespeople, Neolithic villagers and Middle Palaeolithic Neanderthals are less important than the fact that none of them are modern city folk. This is using non-Western societies solely as a mirror to reflect our own identity. And arguing that prehistoric societies must be considered 'civilized' according to civilization's own self-definition is somewhat self-defeating, making them look less capable rather than more.

Take Neolithic dentistry as an example. As Rudgley points out, Neolithic Europeans practised the earliest known dentistry, as scanning electron microscopy of a drilled tooth from a Danish skeleton shows. But there is almost no other evidence at all for oral hygiene in the Neolithic, and skeletal studies show that Neolithic Europeans almost universally suffered severe dental pathologies. In this context, Neolithic 'dentistry' seems a bit like the dog that played checkers: he didn't do it well; the marvel was that he did it at all.

The alternative (which Rudgley pursues *within* many chapters) would be to take societies on their own terms and look for the meaning of each invention within a society rather than checking it off on a list of traits of

'civilization'. Prehistoric people did many weird and wonderful things; the way to read this book is as an entertaining and enlightening account of prehistory's greatest hits. □

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## Happy endings?

### Last Rights: The Struggle over the Right to Die

by Sue Woodman

Plenum Trade: 1998. 293 pp. \$26.95

John Galloway

"It is silliness to live when to live is torment; and then have we a prescription to die when death is our physician." These words, spoken by Roderigo in *Othello*, are the mainspring for *Last Rights*. Sue Woodman's history of 'right-to-die' movements. The title is not just a neat play on words, but goes straight for the jugular. Last rights, not last rites; end of life decisions by doctors have replaced dying with the 'comfort of clergy'. The secular has elbowed aside the spiritual. Why is there still an issue? Roderigo's words seem uncontroversial. The problem now is that modern medicine can keep people alive beyond their 'sell-by date'. On the one hand, people are kept physically alive long after their personalities have been destroyed; on the other, some with their mental faculties intact live lives of dependency and pain.

In reality, 'right-to-die' movements are about the legal right not to be kept alive at all costs, and *in extremis* the right to be killed or helped to commit suicide. Put like this, there are clearly issues. Euthanasia is almost universally illegal, but the line between killing and not keeping alive is one that is easily crossed. For many people, removing a feeding tube from someone on life-support is not so different from giving an 'overdose' of morphine. As a result, medical institutions have often refused to withdraw life-support without court sanction.

Courts tend to uphold the opinions of doctors and their medical institutions against the wishes of patients and their families. Woodman points out that it was not until the Patient Self-Determination Act in 1991 that US federal legislation upheld patients' wishes against those of doctors or medical institutions. This may have been triggered by the Nancy Cruzan case. Severely injured in a car crash, Nancy was kept on life-support for eight years until a judge agreed there was 'clear and convincing' evidence that she would not have wished to live in a 'vegetable state'.

A popular measure, encouraged in the United States by the Hemlock Society, is the 'living will'. This enables people to make decisions about their medical treatment while of 'sound mind' to cover events when

they may not be. Despite the belief that such wills are legally binding, Woodman claims they have often been set aside by doctors.

'Right-to-life' movements have mobilized in opposition to any attempts to legalize euthanasia and physician-assisted suicide. Spearheaded, not surprisingly, by the Catholic Church, they have been supported by organized medicine. Doctors do not wish to be openly associated with death rather than life. Woodman recounts the British Euthanasia Society's failed attempts in the 1930s to have the practice legalized. The Royal Physician, Lord Dawson, argued then that legalizing euthanasia was unnecessary because good physicians already helped their patients die. However, the desirability of doctors acting secretly, above or outside the law, has to be questioned.

In the Netherlands until recently, euthanasia and physician-assisted suicide were illegal, but nevertheless practised with increasing openness. The government would not legalize either, but only prosecuted when doctors failed to abide by strict guidelines. However, fears that the entente between law and medicine was being abused resulted in a series of national studies. The first was commissioned in 1990–91 by a committee chaired by the Attorney-General of the Dutch supreme court and led to new procedures for reporting end-of-life decisions. Further studies were published in 1996 and 1997, which showed little change from the earlier studies. In 20 per cent of deaths, a decision had been taken to withdraw or withhold treatment. Ten per cent had followed drugs given to relieve pain. Less than 3 per cent resulted from euthanasia, and

another 3 per cent from physician-assisted suicide. This type of reliable empirical research about 'end-of-life' decisions achieves two ends. It provides an evidential base for ethical discussion and it brings end-of-life decisions into the open. Openness and transparency must be the best guards against abuse.

Woodman has written a readable book about this legal and ethical minefield. Her journalistic style will not appeal to everyone. However, the book has the advantage of starting with the deaths of real people from which she attempts to draw general conclusions. The result is more powerful than starting with abstract ethical principles and applying these to individual cases. Some deaths, like Nancy Cruzan's, are included because they were pivotal in changing the law. Others, like that of Woodman's aunt, are simply an attempt to tell it 'like it is'.

Woodman also introduces us to the other side of the coin of dying — to crusaders like the retired US pathologist, Jack Kevorkian, who helps people die. Working on the fringes of the law, he has been tried and acquitted several times on charges of unlawful killing. How much of his success in avoiding conviction can be put down to the weakness of the law, and how much to his lawyer, Geoffrey Fieger, is itself a significant question. It reminds us that the law is an imperfect instrument. Enshrining the principle of 'right to die' in statute is one thing; implementing it in a way that delivers what the 'right-to-die' movements want would undoubtedly be another. □

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## Shaping the Earth

Our knowledge of the remarkable and complex forces that have created the Earth we see today continues to grow. Simon Lamb and David

Sington look at these forces and their interactions in *Earth Story: The Shaping of our World* (Princeton University Press, \$29.95).



# The optical counterparts of $\gamma$ -ray bursts

Bernard J. McNamara & Thomas E. Harrison

**The origin of  $\gamma$ -ray bursts—which are among the most energetic events in the Universe—has puzzled astronomers for 25 years. Since 1991, new events have been discovered at a rate of about one per day, but because their positions were poorly determined, the objects responsible for these outbursts could not be identified. Now, following the launch of the BeppoSAX satellite in 1996,  $\gamma$ -ray bursts have been rapidly and accurately located, which has led to the breakthrough discoveries of X-ray and optical counterparts in 1997, and the demonstration that these objects are in general very distant.**

Beginning in 1963, the United States launched a series of satellites that carried detectors of high-energy radiation; these 'Vela' satellites were designed to search for violations of the international nuclear test ban treaty and to collect data on the celestial X-ray,  $\gamma$ -ray and particle background. To the surprise of the Los Alamos researchers who analysed these data, after the false triggers were eliminated, a small sample of high-energy transients remained that appeared to have a cosmic origin<sup>1</sup>. The Vela satellites did not possess the capability to localize these events to better than  $10^\circ$  in the sky, so the sources of these outbursts could not be identified.

As more spacecraft carrying high-energy detectors were launched in the late 1970s and early 1980s,  $\gamma$ -ray burst (GRB) detection times at pairs of widely separated spacecraft were employed to produce error boxes as small as one square arc minute (ref. 2). After intense searches were conducted of these small error boxes, no objects were identified that could be unambiguously associated with the bursts. The source of GRBs remained a mystery.

In April 1991, NASA launched the Compton Gamma-Ray Observatory (CGRO). One of its primary missions was to gather data about GRBs. The main instrument used to detect bursts was the Burst and Transient Source Experiment (BATSE). A group called BACODINE<sup>3</sup> used real-time telemetry from the CGRO to compute and distribute burst locations within seconds of the detection of a GRB. On the basis of theoretical studies that indicated that GRBs would produce post-burst emission at other wavelengths, real-time search programmes were started in Europe and quick-response networks were established in the United States. Although BACODINE provided timely error boxes, they were too large to allow the sources of GRBs to be identified.

With the launch of the Italian–Dutch satellite BeppoSAX in April 1996, a new era of GRB follow-up became possible. For the first time, researchers were provided with both timely and small GRB error boxes. Soon after these new localizations became available, counterparts to the GRBs were discovered at X-ray, optical and radio wavelengths. At least two of these transients were found to be located at extragalactic distances, thus providing the first direct evidence of the GRB distance scale.

## Early GRB research

By 1991, GRB research had evolved into a separate field of high-energy astrophysics. Despite nearly two decades of work, however, some very basic questions remained unanswered. Were GRBs located within our Galaxy or at cosmological distances? What objects produced GRBs? What triggers a GRB, and what mechanism produces its high-energy emission?

In the early 1990s, most researchers believed that the GRBs originated from Galactic neutron stars. This hypothesis was based on two factors: first, the presence of very short (millisecond) structure in the burst's high-energy light curve, suggesting a

compact emission region, and second, the observation of absorption lines in some of the GRB spectra. These absorption features were believed to be cyclotron lines produced by electrons spiralling in the strong magnetic field of a neutron star. If these neutron stars were nearby, then a variety of physical processes could explain the spectra and luminosities of the bursts. Nevertheless, nagging doubts remained about the 'Galactic neutron star' paradigm. Even though relatively few GRBs had been discovered, they appeared to be isotropically distributed in the sky. If they arose from Galactic neutron stars, they should have been concentrated towards the plane of the Galaxy where their progenitors (that is, massive stars) are found. Difficulties existed with both Galactic and extragalactic models of GRBs, and researchers were divided into these two camps. Everyone agreed, however, that the distance scale of the GRBs could be quickly resolved by the discovery of an optical counterpart.

## Searches for the quiescent sites of GRBs

The search for the origin of GRBs focused on the deep imaging of small GRB error boxes at optical, infrared and X-ray wavelengths. The goal of these studies was to identify energetic objects, highly variable sources, or a common class of relatively rare object that could be tied to the GRB through statistical arguments.

Researchers imaged the smallest GRB error boxes to extraordinarily faint optical levels. One study surveyed objects within the error box of GRB790613 to a V-band magnitude of  $m_V = 26$  (very near the limit of ground-based imaging) but found no obvious quiescent candidate objects<sup>4</sup>. Other searches deeply imaged error boxes in the optical and infrared but again no candidate sources were identified<sup>5–7</sup>.

X-ray searches of the smaller GRB error boxes began in 1982. Their objective, like that of the optical studies, was to identify unusual sources and then use probability arguments to establish a connection between them and the GRBs. Although several studies near the sensitivity limit of the X-ray instruments were completed, no compelling GRB host sites were discovered<sup>8</sup>.

## The CGRO era

After nearly a decade of intense effort, frustration was building among workers in this field as study after study failed to identify a plausible source for GRBs. One of the main problems was that these early counterpart searches relied on localizations that were only available long after the burst had occurred. The high-energy emission from a GRB was known to be short-lived. Perhaps quicker follow-up efforts might be able to detect the afterglow of a GRB? This approach became possible with the launch of the CGRO in April 1991.

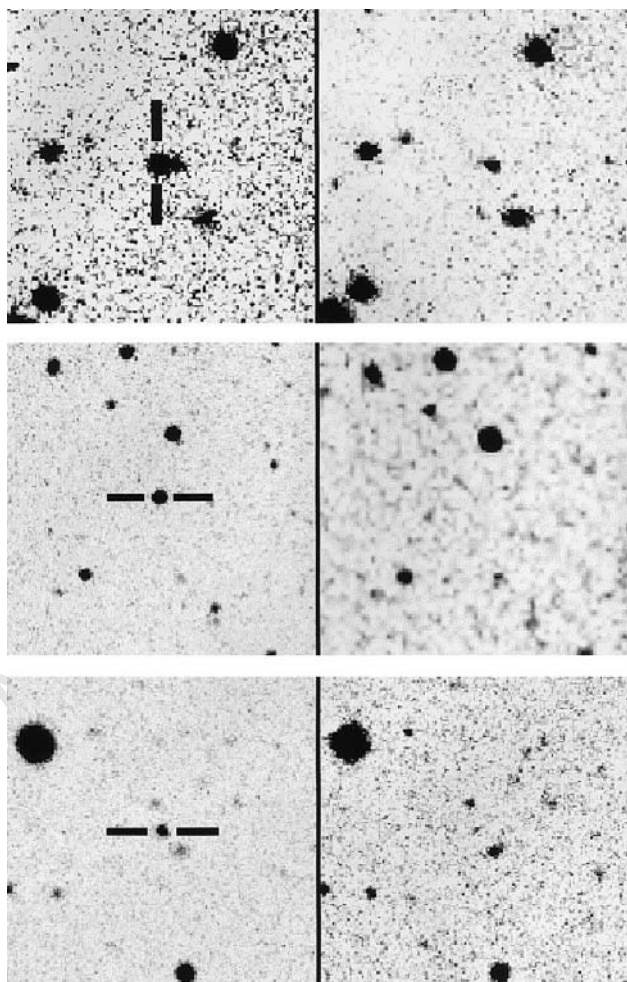
The BATSE/CGRO data soon established that GRBs were isotropically distributed in the sky, strongly suggesting that these objects had a cosmological origin. BATSE also provided real-time



data that were used to quickly compute and distribute GRB localizations. The BATSE/COMPTEL/NMSU Rapid Response Network was established in 1993 to provide a multiwavelength, worldwide, capability to deeply image these positions. Despite several years of effort, no optical counterparts were found to a B-band limiting magnitude of  $m_B \leq 22$  obtained 16 hours after the burst had occurred<sup>9</sup>. European investigators using BATSE positions and cameras with wide fields of view found that no simultaneous optical events brighter than  $m_B \approx 10$  mag occurred<sup>10</sup>. These results were very troubling because they implied that GRBs either did not produce any emission at lower energies, that the burst afterglows were extremely short lived, or that the GRB counterparts were very faint.

## The breakthrough year of 1997

With the launch of BeppoSAX in 1996, GRB counterpart work was revolutionized when it was discovered that GRBs frequently produce relatively long-lasting (of the order of days) X-ray afterglows.



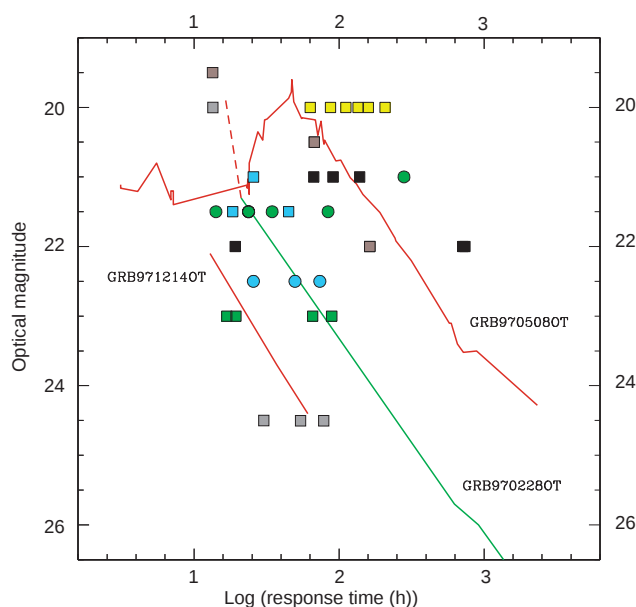
**Figure 1** The optical decay of the transients associated with the bursts GRB970228, GRB970508 and GRB971227. For GRB970228 (top) the images show the discovery observations of the optical transient (OT) (images courtesy of P. Groot, T. Galama and J. van Paradijs). The middle panel shows the images of the transient associated with GRB970508. The left-hand image is for 10 May, when the transient was near maximum brightness<sup>41</sup>, while the right-hand panel is a recent image of the field obtained by the authors using the Astrophysical Research Consortium 3.5-m telescope. The bottom panel shows the decay of the transient for GRB971214<sup>31</sup>. The optical images for GRB980228 are 4 arcmin square, while those for GRB970508 and GRB971214 are  $\sim 1$  arcmin on a side. North is at the top, east is to the left.

BeppoSAX employs an all-sky X-ray detector and two wide-field cameras that view  $20^\circ \times 20^\circ$  (full-width at half-maximum) portions of the sky. The wide-field camera can locate a burst to within a few arc minutes. Observations of the X-ray afterglow with the main BeppoSAX telescope can then produce an error circle with a radius of  $< 1$  arcmin. The timely distribution of these small error circles led to the long-awaited breakthrough in counterpart searches. A second satellite, the Rossi X-ray Timing Explorer (RXTE), launched in December 1995, also produced small GRB error boxes that were searched for GRB counterparts. A brief review of the GRBs localized in 1997 and their counterpart searches is given below.

**GRB970111.** This was the first BeppoSAX GRB error circle produced in 1997. It had an initial radius of  $\leq 10$  arcmin that was shortly reduced to 3 arcmin. No unambiguous counterpart was found using deep observations about a day later, either by BeppoSAX or by optical telescopes that imaged the error box to  $m_B = 23$  mag and R-band magnitude  $m_R = 22.6$  (ref. 11).

**GRB970228.** The X-ray afterglow of this GRB was used by BeppoSAX to produce an error circle of radius 50 arcsec. Using CCD (charge-coupled device) images taken 9 days apart, an object was found that had faded from  $m_V = 21.3$  mag to  $m_V > 24.2$  mag (ref. 12). In Fig. 1 the discovery images of this source are presented. The Hubble Space Telescope (HST) Wide Field and Planetary Camera was used to image this transient 26 days after the burst was detected, and found that it had faded to  $m_V = 25.7$  mag (ref. 13). HST observations taken about 2 weeks later (April 7.2 UT) showed that the rate at which the transient was fading had slowed ( $m_V = 26.0$  mag). Co-addition of the HST images suggested that the optical transient (OT) was located in a morphologically unusual extended source. The light curve of this OT is presented in Fig. 2.

**GRB970402.** A third burst, GRB970402, was detected by BeppoSAX and its error circle was optically imaged to  $m_R = 21.0$  mag within 18



**Figure 2** The optical follow-up observations of  $\gamma$ -ray bursts. The light curves of the three optical transients (OTs) are shown as solid lines (a red line means an R-band light curve, a green line means a V-band light curve). The dashed red line on the V-band light curve for GRB970228 connects to a pre-discovery R-band observation of the GRB970228 OT. Circles and squares correspond to the limiting magnitude of V- and R-band searches, respectively. The coloured symbols correspond to counterpart searches for OTs associated with GRB970111 (black), GRB970402 (blue), GRB970616 (yellow), GRB970815 (green), GRB970828 (grey), and GRB971227 (brown).

hours by the BATSE/COMPTEL/NMSU Rapid Response Network<sup>14</sup>. Although this was the fastest response time for the deep imaging of any BeppoSAX localization, no optical counterpart was found.

**GRB970508.** The fourth BeppoSAX burst of 1997 was GRB970508. Its error circle was optically imaged 5.5 hours after its detection, and a source was found that brightened by one magnitude over the next 24 hours (ref. 15). The R-band light curve for this transient is plotted in Fig. 2. Spectra obtained using the 10-m Keck II telescope revealed that this OT was at a cosmological distance (at redshift  $z \geq 0.8$ ; ref. 16). A flaring radio source, consistent with the position of the OT, was also detected. The variability of the radio source suggested that the OT was small<sup>17</sup>. Unlike the transient for GRB970228, HST observations were unable to find a galaxy associated with this object to  $m_R = 24.5$  mag. Images of the optical counterpart are shown in Fig. 1.

**GRB970616.** This burst was initially localized by BATSE<sup>18</sup>. Its error circle was later reduced to a radius of about 1.5 arcmin by the RXTE, and two previously unknown X-ray sources were found<sup>19,20</sup>. Observations by the satellites ASCA and Rosat suggested that both of these sources were variable<sup>21</sup>. No optical counterpart was identified for this GRB.

**GRB970815.** This source was detected by BATSE and was simultaneously imaged by the All-Sky-Monitor (ASM) of RXTE. The subsequent RXTE/ASM error box was imaged 0.7 and 1.7 days later to a J-band magnitude of 21.0, and  $m_R = 23.0$  mag (refs 22, 23). No fading source was found. ASCA observations, obtained two days after the burst, failed to detect an X-ray source within the RXTE/ASM error box. A radio search by the Very Large Array extending from August 16.6 to 21.1 UT was negative<sup>24</sup>.

**GRB970828.** This GRB was detected by the RXTE/ASM on August 28.74 UT<sup>25</sup>. A refined position, obtained by ASCA, was optically imaged 0.7 days later but no fading source was found<sup>26</sup>. A radio source was identified<sup>27</sup> within the error box that appears to be associated with a galaxy having  $m_B = 20.2$  mag. Temporally coincident optical images of this galaxy, however, show it to be non-variable.

**GRB971214.** This burst was discovered by BeppoSAX on December 14.97 UT with a peak X-ray flux of about 1 Crab (ref. 28). An optical candidate was detected at I-band magnitude  $m_I = 21.2$  on December 15.47 UT<sup>29</sup>. It faded to  $m_I = 22.9$  mag by December 17.46 UT<sup>30</sup>. The colour of this object remained approximately constant during its decline<sup>31</sup>. The galaxy associated with this transient is extremely distant, having redshift  $z = 3.46$  (ref. 32). Images of the decay of the optical transient are shown in Fig. 1, while the light curve is plotted in Fig. 2.

**GRB971227.** This GRB was detected by BeppoSAX on December

27.35 UT but because the satellite's pointing was not optimal, the X-ray source could only be localized to a 10 arcmin error box<sup>33</sup>. This localization was improved to about 1.5 arcmin, 14 hours later, and two previously unknown X-ray sources were found<sup>34</sup>. One of these sources had a stable X-ray flux, whereas the other had faded below the detection limit of BeppoSAX. The latter, fading, source was suggested to be associated with the GRB. An optical counterpart was suggested, but not confirmed for this GRB.

## Overview of 1997 counterpart searches

What have we learned from the detected optical transients? Table 1 summarizes the observational parameters of the 1997 GRBs. Of the six GRBs where an X-ray counterpart was observed, only three had associated OTs. Interestingly, there does not appear to be a correlation between the high-energy flux of the GRB event and the detection of an OT. Both 'bright' and 'faint' GRBs produced optical counterparts, whereas others of similar strength did not. This is not due to a selection effect, because, as Fig. 2 shows, the bursts without optical counterparts were searched as deeply and as rapidly as those that had optical detections. Galactic extinction probably cannot be invoked to explain this result, although the statistics supporting this statement are weak. Except for GRB970402, none of the bursts occurred close to the Galactic plane, and the bursts without counterparts were at as high a Galactic latitude as those with counterparts. The non-detection of GRB970828 to R-band magnitude  $m_R = 24.5$  (25 hours after burst detection) implies that the ratio of the peak X-ray to optical flux  $(F_X/F_{opt})_{peak} \geq 2,700$ . Comparing this result to that for GRB970508 (see Table 1) suggests that real differences in this ratio exist between GRBs. The light curve of the OT associated with GRB970508 also differs from the other two transients (see Fig. 2), and it was also the only one of the three OTs detected at radio wavelengths.

## Theoretical implications

The cosmological distances of GRBs implied by their measured redshifts requires that whatever process creates them releases a tremendous amount of energy. Current models assume that GRBs are associated with compact objects which, because of an infrequent event (for example, a binary neutron-star merger), expel matter at highly relativistic velocities<sup>35–39</sup>. As the expanding shell (often referred to as a fireball) sweeps up material from the ambient environment, it loses energy and radiates at longer wavelengths. GRB models address three different stages in the evolution of the burst. The first deals with the ignition of the fireball itself, and focuses on the burst's short-lived  $\gamma$ -ray radiation. This period corresponds to events that happen close to the ignition site. The second time period corresponds to the expansion of the fireball, and

**Table 1 Parameters of the  $\gamma$ -ray bursts of 1997**

| GRB name | Right ascension*<br>(h min s) | Declination*<br>(° ' ") | $N_H$<br>column<br>( $10^{20} \text{ cm}^2$ ) | Peak flux<br>2–10 keV<br>(Crab) | Counterpart?† |   |     | $(F_X/F_{opt})_{peak}$ |
|----------|-------------------------------|-------------------------|-----------------------------------------------|---------------------------------|---------------|---|-----|------------------------|
|          |                               |                         |                                               |                                 | X             | O | R   |                        |
| 970111   | 15 28 24                      | +19 40 00               | 4.63                                          | 4.0                             | I             | N | N   | –                      |
| 970228   | 05 01 46.7                    | +11 46 53.0             | 15.33                                         | 6.8                             | Y             | Y | N   | $\leq 88,000$          |
| 970402   | 14 50 06                      | –69 20 00               | 2.20                                          | 0.46                            | Y             | N | N/A | –                      |
| 970508   | 06 53 49.5                    | +79 16 19.5             | 5.18                                          | 1.0                             | Y             | Y | Y   | 1,030                  |
| 970616   | 01 18 59                      | –05 24 40               | 4.38                                          | ?                               | I             | N | N/A | –                      |
| 970815   | 16 08 47                      | +81 33 45               | 4.58                                          | 2.0                             | I             | N | N   | –                      |
| 970828   | 18 08 30                      | +59 19 20               | 3.50                                          | 0.756                           | Y             | N | N   | –                      |
| 971214   | 11 56 26.4                    | +65 12 00.5             | 1.61                                          | 1.0                             | Y             | Y | N/A | $\leq 6,900$           |
| 971227   | 12 57 15                      | +59 24 02               | 1.31                                          | 1.8                             | Y             | I | N/A | –                      |

\* Equinox 2000.

† "Y" indicates that a counterpart was detected, "N" means that no counterpart was detected, "I" means that the search was inconclusive, and "N/A" indicates that no search was attempted.

the release of lower-energy photons. The duration of this phase is a few weeks and is believed to produce the optical decays seen in Fig. 2. The third time frame corresponds to the deceleration of the fireball to sub-relativistic velocities. During this period the optical and radio emission decreases in a much more gradual manner.

There are a number of different GRB fireball models; the differences are in the manner in which the fireball is energized (impulsively or continuously), the geometry of the fireball (spherical or beamed), the density of the surrounding medium (constant or decreasing with radius), and the manner in which the fireball dissipates its energy. More observational data will be needed to differentiate among these various possibilities.

## Future progress in GRB follow-up work

The success of counterpart searches has moved the investigation of GRBs beyond a discussion of their distance scale into an era where realistic models are being constructed. The post-GRB emission-decay rates, and the radio scintillation observations of GRB970508, support GRB fireball models. Several outstanding issues remain to be addressed. What is the source environment of GRBs? What set of circumstances leads to the creation of a GRB? What emission mechanisms operate within these systems? Why is there no apparent correlation between the fluence of a GRB and its afterglow?

BeppoSAX should continue to play a significant role in answering the above questions by providing timely, small error boxes. Based on the present burst-detection rate of BeppoSAX, about a half-dozen new X-ray sources associated with GRBs should be localized during the next year. The RXTE should provide a smaller number of additional localizations. If the present statistics continue, about one-third of these events will produce OTs. It appears likely that the deep imaging capability of the HST will be required to reveal the presence of any host galaxies. Unless brighter OTs accompany future events, telescopes of the 10-m class will be required to obtain their redshifts. The extragalactic nature of GRBs is strongly supported by the current data. However, if one includes the recently discovered burst, GRB980425<sup>40</sup>, the measured GRB redshift values cover a wide range ( $z = 0.01, 0.8$  and  $3.4$ ). If GRB980425 is considered a normal GRB, this result provides yet another interesting twist in the GRB saga. □

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# A meteorite from the Cretaceous/Tertiary boundary

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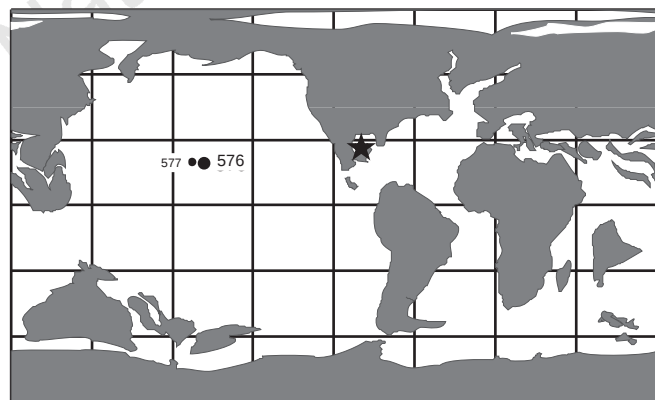
Cretaceous/Tertiary boundary sediments are now widely recognized to contain the record of a large asteroid or comet impact event<sup>1</sup>, probably at the site of the Chicxulub crater on the Yucatan peninsula<sup>2</sup>. After nearly two decades of intensive research, however, much remains unknown about the specific nature of the projectile and of the impact event itself. Here we describe a 2.5-mm fossil meteorite found in sediments retrieved from the Cretaceous/Tertiary boundary in the North Pacific Ocean that we infer may be a piece of the projectile responsible for the Chicxulub crater. Geochemical and petrographic analyses of this meteorite indicate that it probably came from a typical metal- and sulphide-rich carbonaceous chondrite rather than the porous aggregate type of interplanetary dust considered typical of cometary materials<sup>3</sup>. The fact that meteorite survival should be enhanced by impacts at low (asteroidal) velocities<sup>4</sup> also implies that this meteorite had an asteroidal rather than a cometary origin.

Cretaceous/Tertiary (K/T) boundary sediments in DSDP Hole 576 (32° 21.4' N, 164° 16.5' E) are dark-brown, oxidized, pelagic clays, typical of deep-ocean sediments in the North Pacific. The silicate fraction of this sediment is fine-grained (~3 µm) wind-blown dust derived from North America and Asia<sup>5</sup>. 65 Myr ago, this site was located in the central portion of the palaeo-North Pacific basin (Fig. 1), 9,000 km west of the Chicxulub impact structure<sup>2,6</sup>, and thousands of kilometres from the nearest continent. The K/T boundary here has a broad iridium anomaly<sup>7</sup>, shocked quartz grains<sup>8</sup> as large as 190 µm and grains of magnesioferrite spinel<sup>9</sup> (presumably derived from ~250-µm spherules). One sediment sample (Hole 576 core 8, section 1, 50–52 cm) from the base of the iridium anomaly contained an anomalous ~4-mm inclusion of light-brown clay. This inclusion was separated, air-dried, and split open to reveal a 2.5-mm lithic clast (Fig. 2) which we have found is a

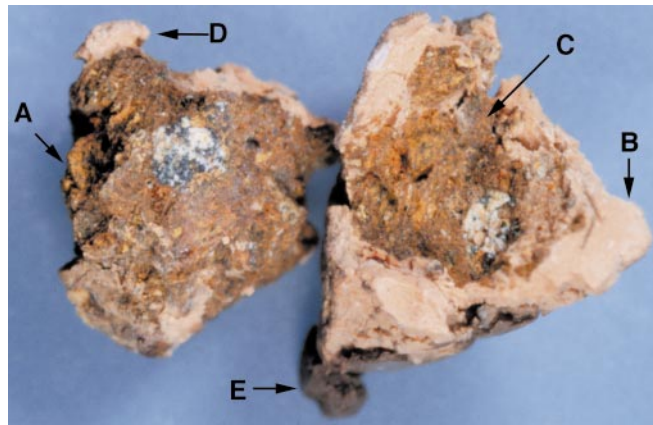
fossil meteorite. The clast has a brecciated texture with a fine-grained matrix enclosing rounded to angular inclusions of various colours, and its surface is peppered with numerous small (<50 µm) opaque grains.

Six chips from separate portions of the meteorite were mounted in polished sections (a total of 2.4 mm<sup>2</sup>) to examine its interior. In polished section, the meteorite is distinguished from the surrounding clays by its high concentrations of iron oxides that ranged from <1 µm to 250 µm in size. Distinct textural domains can be distinguished (Fig. 3a, b). In some areas, clays and oxides are intergrown on a micrometre scale. Sometimes there are sharp borders around regions of nearly pure clay, which often contain large inclusions of iron oxides. The clay-rich inclusion shown in Fig. 3b is interpreted to be a pseudomorphic replacement of pre-existing olivine, and the patches of iron oxides within it are probably pseudomorphs after Fe–Ni metal. For comparison, an olivine grain with metal inclusions from the CM chondrite LEW85001 is shown in Fig. 3c.

Electron-microprobe analyses of the oxides (Table 1) typically total ~90% when Fe is measured as FeO, indicating that the principal oxide is haematite (Fe<sub>2</sub>O<sub>3</sub>). Nickel oxide is detected in most of the larger haematite grains. Although NiO is typically <1%, several grains were found with 2–7% NiO (Table 1). Some haematite contains finely dispersed, submicrometre, Ni–Fe metal and Ni–Fe sulphides (Fig. 3d). Mostly these are too small to analyse in the microprobe, but qualitatively Ni/Fe ratios are always high. Only one metal grain was large enough to analyse quantitatively (Fig. 3d) and yielded a composition of Ni<sub>87</sub>Fe<sub>13</sub>. Microprobe analyses of the clays are difficult because of the hydrous, porous nature of these samples. Even with broad-beam (~10-µm) analyses, totals typically are <85%. The relatively pure clays in oxide-free zones are characterized by high MgO and FeO concentrations, and are compositionally distinct from the surrounding clays of the light-brown rim (Table 1). Typically MgO concentrations range from 6% to 10%, but concentrations as high as 15% have been measured. Based on cation ratios, these clays are probably interlayered glauconite and smectite. The region with large patches of haematite on the left side of Fig. 3a has some intergrown clays with 24% MgO (Table 1) that are identified as saponite. The clays of the light-brown rim are texturally and compositionally similar to the dark-brown clays typical of the bulk sediments. Both contain phosphate grains up to 50 µm in size that are probably fish scale and bone debris. The



**Figure 1** Palaeoreconstruction map. Shown are the locations of DSDP sites 576 and 577 (filled circles) and the Chicxulub impact structure (star) at the time of the K/T boundary impact (65 Myr ago).



**Figure 2** Photograph of separated meteorite and surrounding clays. A fossil meteorite (A) was found encased in light-brown clays (B). Portions of the meteorite still line the interior cavity of the light-brown clays (C), and the white spot in the cavity is a portion of the black and white inclusion at the centre of the meteorite surface. We note that some light-brown clays remain on the surface of the meteorite (D). Typical sediments from the K/T boundary at DSDP Site 576 are dark brown. Small pieces of dark-brown clay (E) can be seen on the bottom of the piece of light brown rim. Largest dimension of the meteorite is 2.5 mm.

**Table 1 Selected electron microprobe analyses**

|                                | Lithic oxide | Lithic oxide | Lithic clay | Lithic clay | Lithic clay | Light-brown rim | Dark-brown clay |
|--------------------------------|--------------|--------------|-------------|-------------|-------------|-----------------|-----------------|
| SiO <sub>2</sub>               | 0.06         | 0.00         | 37.69       | 41.65       | 53.85       | 50.55           | 48.03           |
| TiO <sub>2</sub>               | 0.26         | 0.06         | 0.35        | 0.21        | 0.07        | 0.45            | 0.67            |
| Al <sub>2</sub> O <sub>3</sub> | 0.03         | 0.00         | 12.67       | 11.39       | 3.30        | 16.81           | 15.05           |
| Cr <sub>2</sub> O <sub>3</sub> | 1.52         | 0.23         | 0.42        | 0.35        | 0.36        | 0.00            | 0.06            |
| FeO                            | 86.98        | 82.47        | 20.92       | 12.98       | 4.77        | 7.14            | 6.81            |
| MnO                            | 0.07         | 0.00         | 0.06        | 0.03        | 0.03        | 0.27            | 2.82            |
| MgO                            | 0.21         | 0.57         | 7.80        | 15.57       | 24.06       | 3.73            | 3.62            |
| NiO                            | 0.60         | 5.50         | 0.04        | 0.23        | 0.12        | 0.12            | 0.01            |
| CaO                            | 0.05         | 0.28         | 0.16        | 0.31        | 0.06        | 1.14            | 1.77            |
| Na <sub>2</sub> O              | 0.00         | 0.07         | 1.43        | 2.64        | 0.78        | 1.42            | 1.62            |
| K <sub>2</sub> O               | 0.00         | 0.00         | 3.11        | 2.98        | 0.76        | 4.30            | 4.09            |
| Cl                             | 0.00         | 0.04         | 0.53        | 1.53        | 0.41        | 0.29            | 0.66            |
| Total                          | 89.78        | 89.22        | 85.18       | 89.87       | 88.57       | 86.22           | 85.21           |

All values in per cent.

**Table 2 Trace-element concentrations in bulk samples**

|                             | Lithic clast | Light-brown clays | Dark-brown clays | CI*    | CV*    |
|-----------------------------|--------------|-------------------|------------------|--------|--------|
| Sc ( $\mu\text{g g}^{-1}$ ) | 49           | 38                | 31               | 5.8    | 11.4   |
| Cr ( $\text{mg g}^{-1}$ )   | 6.54         | 0.11              | 0.05             | 2.7    | 3.6    |
| Fe ( $\text{mg g}^{-1}$ )   | 413          | 54                | 54               | 182    | 237    |
| Co ( $\mu\text{g g}^{-1}$ ) | 76           | 51                | 182              | 508    | 659    |
| Ni ( $\mu\text{g g}^{-1}$ ) | 1,370        | 216               | 389              | 10,700 | 13,400 |
| Zn ( $\mu\text{g g}^{-1}$ ) | 565          | 176               | 203              | 312    | 116    |
| As ( $\mu\text{g g}^{-1}$ ) | 312          | 44                | 17               | 1.8    | 1.5    |
| Rb ( $\mu\text{g g}^{-1}$ ) | <60          | 112               | 89               | 2.2    | NA     |
| Sb ( $\mu\text{g g}^{-1}$ ) | 21           | 3.9               | 2.7              | 0.15   | 0.08   |
| Cs ( $\mu\text{g g}^{-1}$ ) | 1.3          | 7.0               | 6.2              | 0.18   | NA     |
| La ( $\mu\text{g g}^{-1}$ ) | 11           | 165               | 159              | 0.24   | 0.47   |
| Sm ( $\mu\text{g g}^{-1}$ ) | <0.5         | 0.7               | 0.7              | 0.02   | NA     |
| Ir ( $\text{ng g}^{-1}$ )   | 690          | 38                | 2.6              | 460    | 771    |
| Au ( $\text{ng g}^{-1}$ )   | 213,000      | 3,500             | <10              | 144    | 143    |
| Th ( $\mu\text{g g}^{-1}$ ) | 0.8          | 8.7               | 8.6              | 0.03   | NA     |

NA, not available.

\* Concentrations in CI (ref. 29) and CV (ref. 31) chondrites provided for comparison.

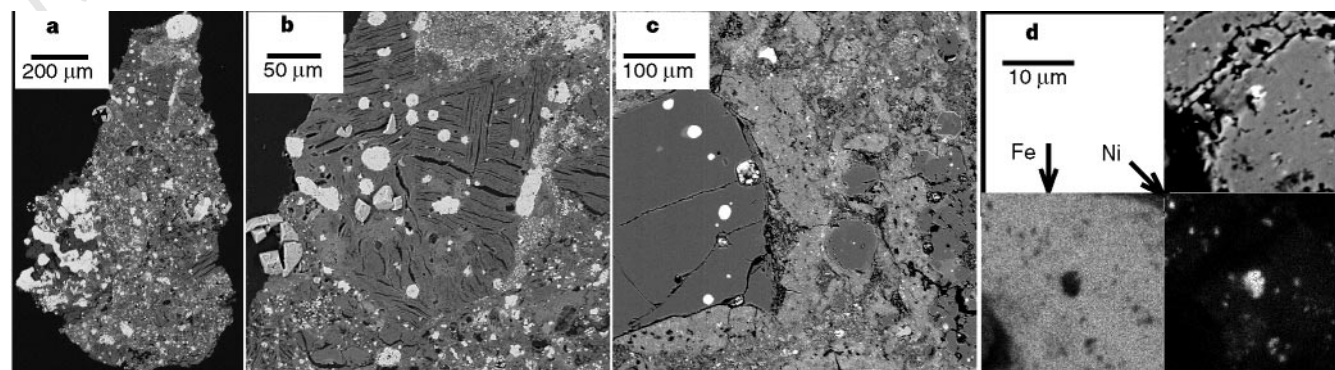
main difference between these two samples is the relative absence of MnO in the light-brown rim.

Neutron activation analyses (Table 2) also show the compositional difference of the fossil meteorite from the surrounding sediments. Compared to the dark-brown sediments of the K/T boundary, this clast is highly enriched in the siderophile elements Ir, Au and Cr and the commonly chalcophile elements As and Sb, while it is depleted in lithophile elements such as Cs, La, Sm and Th. Although the concentrations of lithophiles are significantly higher than in chondritic materials, this may result from contamination from the rim, which was not entirely removed from this sample (Fig. 2). The light-brown clays that rim the clast are also highly enriched in Ir, Au and As relative to the brown clays, but to a much lesser degree. However, the lithophile contents of the light-brown and dark-brown clays are similar to each other, an observation consistent with the petrographic and microprobe data.

For comparison, the best documented occurrence of a fossil meteorite is that of Brunflo, a 10-cm chondritic clast recovered from an Ordovician limestone<sup>10,11</sup>. Brunflo is remarkable in that detailed textures of meteoritic chondrules have been preserved, despite nearly complete chemical exchange with surrounding carbonate sediments. The primary mafic silicates, metal and sulphides of Brunflo were altered to calcite, barite, illite and a host of trace mineral phases. The fossil meteorite from DSDP Hole 576 also appears to have retained primary textures, but there is no reason to believe that any primary mineralogy has been preserved. The traces of Ni-rich metal and sulphide are probably not primary phases, but

residues from alteration of pre-existing Fe–Ni metal and sulphides. A similar process has been described in cosmic spherules from Pacific sediments, where oxidation of the metallic core in an iron sphere<sup>12</sup> formed a metallic residue with 89% Ni. Although saponite is a common mineral in some carbonaceous chondrites<sup>13</sup>, having formed by aqueous alteration of ultramafic phases, its presence in this rock could also be the result of secondary alteration following burial.

Chemical alteration has certainly severely perturbed the composition of the meteorite reported here. Remarkably, Ir concentrations remain within the range typical of chondritic meteorites, and Fe and Cr concentrations remain within a factor of two of these values (Table 2). As meteorites are not compositionally homogenous on a millimetre scale, the abundances of these three elements could be considered a match with chondritic abundances. The low concentrations of Ni and Co are readily explained by loss during diagenetic alteration. For example, weathered samples of Maralinga, an Australian CK chondrite, have suffered 70% loss of Ni and 40% loss of Co relative to unweathered CK chondrites, while concentrations of Cr, Fe and Ir were relatively unaffected<sup>14</sup>. There is no simple explanation for the 1,000 times enrichment of Au over chondritic values. Apparently Au is also mobilized during alteration, as a 60% loss of this element was observed in Maralinga. The only explanation that we can propose for the extreme Au enrichment is that during early diagenesis of the initial K/T boundary ejecta deposit, Au was mobilized and the reducing microenvironment of the meteorite fragment acted as a sink for Au diffusing



**Figure 3** Backscatter electron images of polished sections from the fossil meteorite. Relict textures of mineral phases that have been replaced by haematite (white grains) and clays (dark-grey matrix) can be seen. **a**, The region with large hematite grains on the left of the image is a portion of the black and white inclusion on the surface of the meteorite in Fig. 2. Haematite in this inclusion contains 1–5% NiO and traces of micrometre-sized nickel sulphides. Dark clays in the centre of this inclusion are saponite. **b**, Expanded view of a region from the top of **a**, which shows a clay-rich zone with sharp boundaries and spheroidal inclusions of

haematite. This is probably a pseudomorph after euhedral olivine in a fine-grained matrix. Equant bright grey grains that stand out in relief at the left-centre of the image are NaCl crystals that crystallized from interstitial salts after the section was made. **c**, Euhedral olivine with metal inclusions in fine-grained matrix from the CM chondrite LEW85001. **d**, Backscatter (top) and X-ray images for Fe and Ni (bottom) from a haematite grain with metal inclusions. The large Ni-rich spot has a composition of Ni<sub>87</sub>Fe<sub>13</sub>.

through sediment pore waters. This admittedly far-fetched scheme requires all of the Au deposited in ejecta over at least 30 cm<sup>2</sup> of the ocean floor to have diffused into this 2.5-mm particle. There is evidence for chemical diffusion at least on a millimetre-scale. The clays surrounding the meteorite are light brown because they lack the hydrogenous manganese oxides that are ubiquitous in pelagic clays and which give them their dark-brown colour. During diagenetic alteration of metal and sulphides within the meteorite, a zone of reducing conditions in the surrounding sediments must have developed, reducing Mn to the soluble Mn<sup>2+</sup> (ref. 15) which could then diffuse outwards. Glauconite, which we infer to be a principal clay mineral in the altered meteorite, is also known to form only under reducing conditions<sup>16</sup>.

The Ir anomaly in DSDP Hole 576 was smeared across at least 30 cm by bioturbation<sup>7</sup>. In such slowly accumulating clays<sup>5</sup> this interval could represent as much as 0.5 Myr of sedimentation, so it is impossible to state with certainty that this object accreted at the same moment as the K/T projectile. Interplanetary dust from the asteroid belt is an unlikely source because it should be strongly biased against particles this large which are destroyed by collisions before solar wind induced drag can decelerate them into Earth-crossing orbits<sup>17</sup>. In any case, over 0.5 Myr, only ~2 × 10<sup>16</sup> g of interplanetary dust should accrete to the Earth, an amount which is <4% of the mass of the 10-km chondritic asteroid proposed for the K/T boundary<sup>1</sup>. Dust produced by a significant comet shower<sup>18</sup> is a potential source, but there is no independent physical evidence that such an event occurred 65 Myr ago. The K/T projectile itself is capable of producing unmelted meteorite fragments and should be considered the most plausible source. Numerical simulations of the Chicxulub impact show that vertical impact of a 10-km projectile at asteroidal velocities could result in as much as 10% of the projectile experiencing shock pressures below the melting point<sup>4</sup>. At more typical angles approaching 45°, even more material could survive. This has also been demonstrated in laboratory experiments of oblique impacts<sup>19</sup>, which have been discussed as the possible cause of asymmetries in the Chicxulub crater<sup>20</sup>. Also, a late Pliocene hypervelocity impact is known to have produced millimetre- to centimetre-sized unmelted meteorites<sup>21</sup> so the potential for meteorite survival is an established fact.

The fossil meteorite described in this study is, to our knowledge, the first K/T boundary sample with sufficient information from textural and chemical data to make inferences concerning its origin. Shuryatz *et al.*<sup>22</sup> reported micrometre-sized Ir nuggets in impact melt rocks from Chicxulub, and speculated that they might be derived directly from the projectile. Robin *et al.*<sup>23</sup> reported ~250-μm spheroidal debris with chondritic Ir concentrations and shapes reminiscent of partially melted interplanetary dust. These were in K/T sediments from DSDP Site 577, only 500 km west of Site 576. They made no specific interpretation of the source materials, other than that they were chondritic. The fossil meteorite from DSDP Hole 576 appears to be from (1) a chondritic meteorite with (2) significant amounts of metal and sulphide (4–8%), (3) large inclusions (>200 μm) of mafic minerals that also contained metal, and (4) 30–60% fine-grained matrix. The known meteorite groups that best fit these criteria could be the CV, CO and CR carbonaceous chondrites<sup>24–26</sup>. Type CM carbonaceous chondrites are possible, but they typically contain only 1–3% opaque minerals<sup>27</sup>. Unequilibrated ordinary chondrites are a less likely source because they have only 15% fine-grained matrix<sup>28</sup>. Carbonaceous chondrites constitute only a few per cent of observed meteorite falls<sup>29</sup>, but they may be an important component of the asteroid belt, as most of the Antarctic micrometeorites have characteristics typical of CM and CR chondrites<sup>30</sup>. □

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## Coherent quantum control of two-photon transitions by a femtosecond laser pulse

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Coherent quantum control<sup>1–3</sup> has attracted interest as a means to influence the outcome of a quantum-mechanical interaction. In principle, the quantum system can be steered towards a desired state by its interaction with light. For example, in photoinduced transitions between atomic energy levels, quantum interference effects can lead to enhancement or cancellation of the total transition probability. The interference depends on the spectral



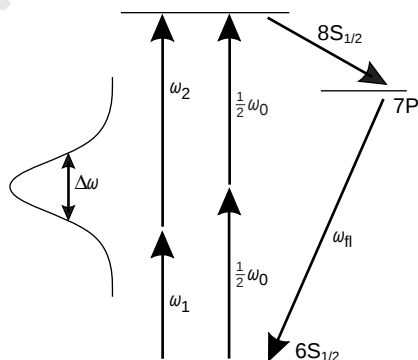
phase distribution of the incident beam; as this phase distribution can be tuned, the outcome of the interaction can in principle be controlled. Here we demonstrate that a femtosecond laser pulse can be tailored, using ultrashort pulse-shaping<sup>4–7</sup> techniques, to control two-photon transitions in caesium. By varying the spectral phases of the pulse components, we observe the predicted cancellation of the transitions due to destructive quantum interference; the power spectrum and energy of these ‘dark pulses’ are unchanged. We also show that the pulse shape can be modified extensively without affecting the two-photon transition probability.

Although schemes of coherent control can involve continuous waves, many proposals have been made that involved ultrashort optical pulses. For example, coherent control of molecular systems with a sequence of short pulses could allow the selective manipulation of molecular structures<sup>8</sup>, including breaking specific bonds and changing a reaction path. Most of these schemes involve exciting the system with short pairs of pulses<sup>9–14</sup>. Such control by pulse pairs was also demonstrated for various one-photon<sup>15,16</sup> and two-photon transitions in two-level systems<sup>17–20</sup>.

Consider the two-photon interaction of an ultrashort pulse with a field  $e(t)$  with a two-level atom. If the interaction is non-resonant (that is, a transition with no intermediate states), the probability of inducing a transition to the excited state by the pulse is proportional to:

$$S_2 = \left| \int E(\omega_0/2 + \Omega) E(\omega_0/2 - \Omega) d\Omega \right|^2 = \left| \int A(\omega_0/2 + \Omega) A(\omega_0/2 - \Omega) \exp \left\{ i[\Phi(\omega_0/2 + \Omega) + \Phi(\omega_0/2 - \Omega)] \right\} d\Omega \right|^2 \quad (1)$$

where  $E(\omega) = A(\omega)\exp[i\Phi(\omega)]$  is the Fourier transform of  $e(t)$ , and  $A(\omega)$  and  $\Phi(\omega)$  are the spectral amplitude and the spectral phase distribution, respectively. Equation (1) reflects the fact that two-photon transitions occur for all pairs of photons with frequencies  $\omega_1$ ,  $\omega_2$  (with  $\omega_1 + \omega_2 = \omega_0$ ), and  $\omega_1$ ,  $\omega_2$  lie within the spectrum of the exciting pulse, as shown schematically in Fig. 1. Hence, all frequency components of a single pulse contribute to the two-photon transition probability, which can be controlled by tailoring the spectral phases of the pulse.



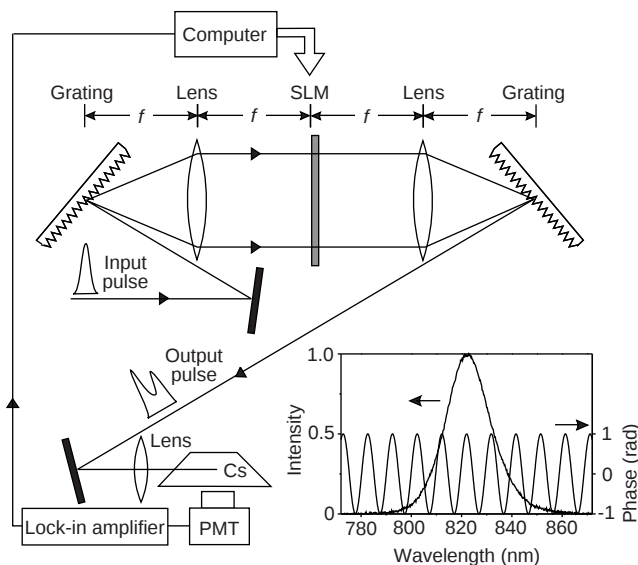
**Figure 1** Schematic diagram of the energy levels of the  $6S_{1/2} - 8S_{1/2}$  two-photon transitions in Cs. Two-photon transitions occur for all pairs of photons  $\omega_1$ ,  $\omega_2$  (with  $\omega_1 + \omega_2 = \omega_0$ ), where  $\omega_0$ , which corresponds to 411 nm, is the energy of the transition. The fluorescence signal  $\omega_f$  corresponds to  $\sim 460$  nm. The power spectrum of the excitation pulse is shown schematically at the left. Note that each of the two levels is split into two hyperfine states. Only two transitions are allowed, one from each of the sublevels of the ground state to the corresponding excited state. In our experiments, all signals were shorter than a few picoseconds, which is too short to observe any dynamics between the two transitions; we therefore consider the ground and excited states as single states.

For a given power spectrum  $A^2(\omega)$ , it is obvious that  $S_2$  is maximized by the transform limited pulse, that is, a pulse having the minimum time duration with  $\Phi(\omega) = 0$ . For a pulse with the same power spectrum, but having any antisymmetric spectral phase distribution around the two-photon transition frequency  $\omega_0/2$ , that is  $\Phi(\omega_0/2 + \Omega) = -\Phi(\omega_0/2 - \Omega)$ , the two-photon transition probability is independent of the spectral phase, and is identical to that of the transform limited pulse. But it is obvious that such an antisymmetric spectral phase affects the pulse shape. In fact, the pulse can be spread in time into a very small amplitude without affecting the two-photon transition probability.

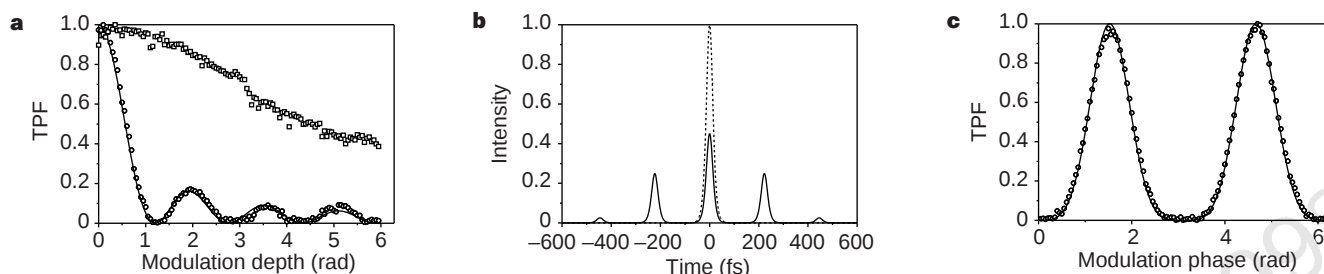
Although antisymmetric phase distributions do not affect the two-photon transition probability, other phase distributions can be tailored to cancel the two-photon transition probability. Such so-called dark pulses, which could come in various temporal shapes, induce no net two-photon transitions, and are the appropriate coherent superposition of optical frequencies that cancels two-photon absorption. They are analogous to dark states, which are coherent superpositions of quantum states that do not absorb resonant light.

To verify our predictions experimentally, we considered the two-photon transitions between the  $6S_{1/2}$  and the  $8S_{1/2}$  levels of atomic caesium, induced by a femtosecond pulse as shown in Fig. 1, where  $\omega_0/2$  corresponds to 822 nm. Each of the excited atoms decays spontaneously to the ground level through the 7P level, so that the two-photon transition can be directly observed through the measurement of the two-photon fluorescence signal at  $\omega_f$ , which corresponds to  $\sim 460$  nm.

The layout of our experimental system, shown in Fig. 2, is composed of a programmable pulse shaper, a Cs gas cell, a photomultiplier and a lock-in amplifier. We tuned our mode-locked Ti-sapphire laser, to produce 31-fs full-width at half-maximum (FWHM) transform-limited  $\text{sech}^2$  intensity pulses, centred at 822 nm, at the output of the pulse shaper. The programmable pulse shaper<sup>7</sup> was composed of a pair of diffraction gratings with



**Figure 2** Experimental system for coherent quantum control of two-photon transitions. The system is composed of a dynamic pulse shaper, a programmable liquid-crystal spatial light modulator used as a spatial filter, a Cs gas cell and a photomultiplier (PMT) and a lock-in amplifier. The computer was used for reading the fluorescence signal and for updating the modulator. The inset shows the normalized power spectrum of a typical input pulse with a FWHM of 23 nm, and a typical phase modulation with  $\Phi(\Omega) = \alpha \cos(\beta\Omega)$ ,  $\alpha = 1$  and  $\beta \approx 220$  fs. In all experiments, an additional constant-phase filter was applied to the modulator, to account for residual dispersion introduced by the pulse shaper, including that of the modulator itself.



**Figure 3** Experimental and calculated results for coherent quantum control of two-photon transitions in Cs gas by a periodic phase distribution  $\Theta(\Omega) = \alpha \cos(\beta\Omega + \varphi)$ . In all cases,  $\beta \approx 220$  fs, which corresponds to about two periods of phase modulation within the FWHM spectral width of the pulses. **a**, Experimental (circles) and calculated (line) normalized two-photon fluorescence (TPF) signal as a function of the modulation depth  $\alpha$  for  $\varphi = 0$ . Also shown are experimental results (squares) for antisymmetric phase distribution  $\Theta(\Omega) = \alpha \sin(\beta\Omega)$ , as a

function of  $\alpha$ . **b**, The calculated normalized temporal intensity distribution of the dark pulse corresponding to  $\alpha = 1.2$  and  $\varphi = 0$  (solid line) and the transform-limited 31-fs  $\text{sech}^2$  intensity input pulse having the same power spectrum (dashed line). **c**, Experimental (circles) and calculated (line) fluorescence signal for phase distribution  $\Theta(\Omega) = \alpha \cos(\beta\Omega + \varphi)$  as a function of the modulation phase  $\varphi$  for  $\alpha = 1.2$ .

1,200 lines  $\text{mm}^{-1}$ , and a pair of achromatic lenses with a 100-mm focal length. Briefly, the first lens and grating spatially map the complex spectrum of the input pulse at the Fourier plane, where a spatial filter is inserted. The second lens and grating reassemble the spectral components to form a modified time-shaped pulse. A one-dimensional programmable liquid-crystal spatial light-modulator array, composed of 128 computer-controlled discrete phase elements was placed at the Fourier plane of the shaper, and was used as a dynamic filter for spectral phase manipulation of the pulses. The shaped output pulses were focused into the Cs gas cell, and the fluorescence signal was filtered optically and detected by the photomultiplier.

To demonstrate coherent control of the transition, we modified the phases of the spectral components of the input pulse by applying spectral phase-only filters of the form  $H(\Omega) = \exp(i\Theta(\Omega))$  to the modulator, where  $\Theta$  is the frequency deviation from  $\omega_0/2$ , and measured the fluorescence signal as a function of the parameters of the filters. Note that a phase-only filter does not affect the pulse energy or power spectrum. We limit the discussion here to periodic spectral phase distributions with  $\Theta(\Omega) = \alpha \cos(\beta\Omega + \varphi)$ , where  $\alpha$  and  $\beta$  are the modulation depth and frequency modulation, respectively, and  $\varphi$  is the modulation phase (see Fig. 2, inset, for a typical phase modulation). In all the experiments below,  $\beta \approx 220$  fs, which corresponds to about two periods of phase modulation over the spectral width of the pulses. The results seem not to be sensitive to this value.

First, we applied a symmetric spectral phase  $\Theta(\Omega) = \alpha \cos(\beta\Omega)$ , and measured the fluorescence signal as a function of the modulation depth  $\alpha$ . The experimental results are presented in Fig. 3a, together with the theoretical curve calculated from equation (1) with  $E(\omega_0/2 + \Omega) = \text{sech}(1.76\Omega/\Delta\omega) \exp[i\alpha \cos(\beta\Omega)]$ , where  $\Delta\omega$  is the FWHM bandwidth of the power spectrum. We note a strong variation in the fluorescence signal as  $\alpha$  is increased. The fluorescence vanishes at a few values of the modulation depth, the first is near  $\alpha = 1.2$ ; these are dark pulses that induce no two-photon transitions. The calculated temporal distribution of the first dark pulse is shown in Fig. 3b, together with the transform-limited input pulse having the same power spectrum. Because of the periodicity of the spectral phase, this dark pulse is a particular sequence of pulses. Other dark pulses are obtained for higher values of  $\alpha$ . The vanishing of the fluorescence signal near  $\alpha = 1.2$  is related to the first zero of the Bessel function  $J_0(2\alpha_0) = 0$ . For a square uniform spectrum of width  $\Delta\omega$  it can be shown analytically that  $S_2 = 0$  in equation (1) for  $\beta = 2\pi/\Delta\omega$  and  $\alpha_0 = 1.2024$ .

Also shown in Fig. 3a are experimental results with antisymmetric spectral phase  $\Theta(\omega) = \alpha \sin(\beta\Omega)$ . Although theory predicts a constant fluorescence signal, we measure a slow decrease in the signal with increasing  $\alpha$ . We attribute this discrepancy to inherent loss and

imperfect antisymmetry due to the use of discrete phase elements in the modulator. But note that for  $\alpha = 1.2$ , the fluorescence intensity drops only by a few per cent for this antisymmetric filter, whereas it vanishes for the symmetric filter. In the time domain, the corresponding fields differ only in their phase structure; that is, their intensities  $|e(t)|^2$  are practically identical. It is this phase structure that leads to the markedly different outcome of the quantum mechanical process. Antisymmetric phase modulation generates a sequence of pulses as in Fig. 3b that are all in a single quadrature of the field, whereas symmetric modulation generates pulses that alternate between the two quadratures. Pulses of different quadratures lead to destructive interference of the two-photon transition probability<sup>17–19</sup> and, at the appropriate ratio, to its complete cancellation.

To emphasize the importance of symmetry, we measured the fluorescence signal as a function of the modulation phase  $\varphi$  for  $\alpha = 1.2$ . This allows monitoring of the transition probability as the spectral phase distribution is continuously transformed from symmetric to antisymmetric. The experimental and theoretical results are presented in Fig. 3c. Note that simply by shifting the phase-modulation filter across the spectrum, affecting neither the total power nor the power spectrum of the pulse, we were able to control the fluorescence yield by a factor of better than 1 in 200. This is a very impressive demonstration of the effectiveness of quantum interference to achieve coherent control of a quantum-mechanical process. Note also that all theoretical and experimental results are presented without adjustable parameters. The excellent agreement between the experimental results and the simple theory outlined above reflects the fundamental role of quantum interference in two-photon absorption.

Although we could demonstrate remarkable agreement between theory and experiment for a two-level system, two-photon processes in molecular and other complex quantum systems are usually too complicated to model exactly. Nevertheless, we expect that coherent control in such complex systems can be achieved by introducing adaptive ultrashort-pulse-manipulation techniques<sup>3,21–25</sup> that optimize the spectral phases to approach a goal without any prior knowledge of the system.

The principles presented here can be extended straightforwardly to many other multiphoton interactions of ultrashort pulses with matter, including Raman transitions, and work in this direction is in progress. We expect that these findings will open a wide new area for theoretical and experimental work, as well as possible applications in nonlinear spectroscopy and in atomic and molecular physics. □

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## Observation of a square flux-line lattice in the unconventional superconductor $\text{Sr}_2\text{RuO}_4$

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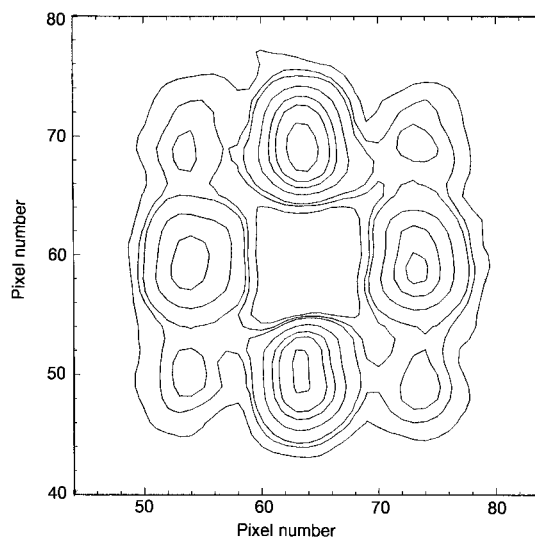
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The phenomenon of superconductivity continues to be of considerable scientific and practical interest. Underlying this phenomenon is the formation of electron pairs, which in conventional superconductors do not rotate about their centre of mass ('s-wave' pairing; refs 1, 2). This contrasts with the

situation in high-temperature superconductors, where the electrons in a pair are believed to have two units of relative angular momentum ('d-wave' pairing; ref. 3 and references therein). Here we report small-angle neutron-scattering measurements of magnetic flux lines in the perovskite superconductor  $\text{Sr}_2\text{RuO}_4$  (ref. 4), which is a candidate for another unconventional paired electron state—'p-wave' pairing, which has one unit of angular momentum<sup>5–7</sup>. We find that the magnetic flux lines form a square lattice over a wide range of fields and temperatures, which is the result predicted by a recent theory<sup>8,9</sup> of p-wave superconductivity in  $\text{Sr}_2\text{RuO}_4$ . This theory also indicates that only a fraction of the electrons are strongly paired and that the orientation of the square flux lattice relative to the crystal lattice will determine which parts of the three-sheet Fermi surface of this material are responsible for superconductivity. Our results suggest that superconductivity resides mainly on the 'γ' sheet<sup>9</sup>.

Strontium ruthenate (SRO) has nearly two-dimensional metallic properties, with a well-established Fermi surface<sup>10–13</sup> consisting of three sheets (α, β, γ). Non-s-wave superconductivity in this material is implied by the strong suppression of the superconducting transition temperature ( $T_c$ ) below its maximum value of ~1.5 K by non-magnetic impurities, which thus act as pair-breakers<sup>7</sup>. Noting that the γ-sheet of the SRO Fermi surface is mainly derived from different Ru orbitals than are the α- and β-sheets, Agerter *et al.*<sup>8</sup> have argued that pairing interactions will only weakly couple the different orbitals. They propose that p-wave superconductivity will be primarily present on either the γ-sheet, or the α- and β-sheets, with weak superconductivity on the other. This argument has been recently extended by Agerter<sup>9</sup>, who has shown that the flux-line lattice (FLL) structure for field perpendicular to the  $\text{RuO}_2$  planes is very likely to be square, and that the orientation of the square flux lattice relative to the crystal lattice indicates which sheet(s) of the Fermi surface are primarily responsible for superconductivity. Thus, by observations of the flux lattice, we can gain important information about superconductivity in this material.

Neutron-diffraction patterns were obtained from the FLL in a sample of SRO (see Methods). A contour plot of a typical result is



**Figure 1** Contour plot of FLL diffraction pattern. With the geometry of our experiments, the diffracted neutrons form an image in the multidetector of the reciprocal lattice of the FLL: the central region of the detector has been masked. A field of 20 mT was applied parallel to **c** above  $T_c$ ; data taken above  $T_c$  were subtracted from that obtained after cooling to 100 mK. The crystal **a** and **b** directions are horizontal and vertical in this figure. The sample shape causes the vertical spots to have different intensities from the horizontal ones, as discussed in Methods. The elongated shape of the spots reflects the shape of the exit aperture of the neutron guide.



shown in Fig. 1. The most notable feature of this pattern is that it is square, with the spots aligned with the **a**, **b** crystal axes, and that it certainly does not represent a triangular FLL. It should be noted that in addition to the  $\{h, k\} = \{1, 0\}$ -type diffraction spots, higher-order  $\{1, 1\}$  spots expected from a square FLL are observed in the expected positions; the intensity of the  $\{1, 1\}$  spots is far too large for them to arise from multiple scattering. In Fig. 2, we show those regions of the  $B$ - $T$  (magnetic induction-temperature) plane where we have established the existence of a square FLL in SRO; shortage of beamtime or weakness of the diffracted signal prevented a complete coverage of the relevant area of the plane, but we have no evidence of any departure from the square lattice structure. Of particular note is the result at 5 mT, which was obtained using an incident wavelength of 30 Å to detect the very long intervortex spacing of 0.64 μm. To our knowledge, this is the lowest-field FLL ever investigated by small-angle neutron scattering (SANS), and shows that there can be an overlap between this technique and decoration. A square FLL in SRO is also indicated by muon spin rotation (μSR) experiments in a purer sample than ours (G. M. Luke, personal communication), and in a less pure one<sup>14</sup>. The cumulative evidence of all these experiments is that in SRO a square FLL is widespread, and possibly intrinsic to its superconductivity.

It should be emphasised that with the field along the four-fold axis of the tetragonal structure of SRO, the original London or Ginzburg–Landau (GL) theories are necessarily isotropic<sup>15</sup>, and hence predict a triangular rather than a square FLL. However, if non-local terms are added to the supercurrent response in the London theory<sup>16</sup>, or higher-order gradient terms are added to the GL theory<sup>17</sup>, then square FLLs can occur. Such theories tend to give a triangular flux lattice as  $T$  tends to  $T_c$ , where non-local effects become unimportant, and also at low magnetic inductions at all temperatures, because the response of any superconductor at long distances tends towards the London response. It appears that these approaches can explain the FLL transformations and square lattices seen in borocarbide superconductors in terms of Fermi surface anisotropy: see refs 17 and 18, and references therein. In general such effects are expected to be stronger in low- $\kappa$  superconductors, which will have stronger interactions between the cores of adjacent flux lines. (The Ginzburg–Landau parameter is defined by  $\kappa = \lambda/\xi$ , where  $\lambda$  is the magnetic penetration depth and  $\xi$  is the coherence

length.) It should also be noted that unconventional superconductivity is not necessarily implied by a square FLL: such a structure was also observed many years ago in a low- $\kappa$  lead alloy<sup>19</sup>. However, another class of theories can also give rise to non-triangular lattices: these are extended GL theories, with more than one order parameter. For instance,  $d$ -wave superconductivity (with an  $s$ -wave component) in copper oxides is also expected to give rise to square FLLs<sup>20</sup>. Another example is the recent theory of  $p$ -wave superconductivity in SRO (ref. 9), which also leads to a square FLL. Using that theory (or indeed, a non- $p$ -wave theory<sup>16</sup>), the relative orientation of the FLL and crystal lattice observed here implies that superconductivity in SRO resides principally in the  $\gamma$ -band electrons. This is not too surprising: we would expect this band to have the strongest pairing interaction, as it has the largest mass enhancement<sup>13</sup>.

To obtain further information about the properties of the superconductor, it is necessary to understand the intensity of the FLL signal, or equivalently, the magnitude of the Fourier components  $|F_{hk}|$  of the magnetic field, described in Methods. In a high- $\kappa$  superconductor, the London model is appropriate, and it gives  $|F_{hk}|$  directly in terms of the temperature-dependent penetration depth,  $\lambda(T)$ , and hence the superfluid density,  $n_s(T)$ :

$$F_{hk}^{\text{London}} = \frac{B}{1 + q_{hk}^2 \lambda^2(T)}, \quad \text{with } \frac{1}{\lambda^2(T)} \propto n_s(T) \quad (1)$$

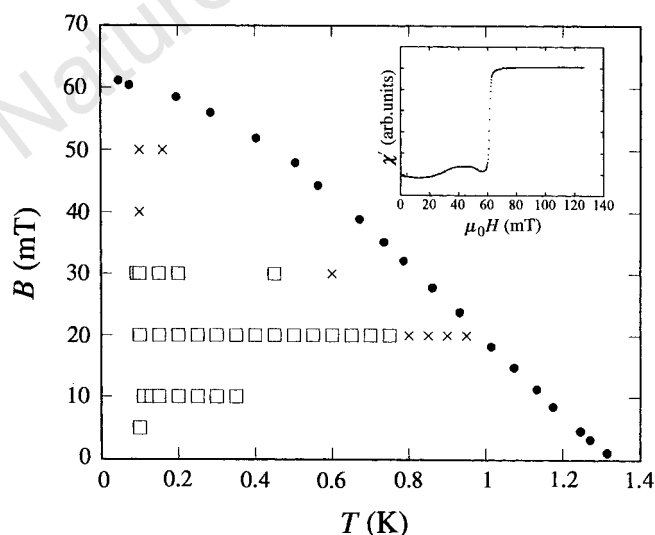
Here  $\mathbf{q}_{hk}$  are the reciprocal lattice wavevectors of the FLL. In a low- $\kappa$  superconductor such as SRO, this will be modified by vortex core overlap. We choose to represent this by using the exact solution of the GL equations, calculated by the method of Brandt<sup>21</sup>. This gives  $F_{hk}^{\text{GL}}/F_{hk}^{\text{London}}$  as a function  $f(B/B_{c2})$ , where  $f$  is weakly  $\kappa$ -dependent. In order to use this approach, we need the upper critical field  $B_{c2}$  (and the coherence length  $\xi$  from  $B_{c2} = \Phi_0/2\pi\xi^2$ , where  $\Phi_0$  is the flux quantum), which was determined as described in Methods and is plotted in Fig. 2.

Assuming the validity of the core overlap correction, we calculated a self-consistent set of  $\lambda$  and  $\kappa$  from the average integrated intensity of the horizontal spots. At 20 mT and 100 mK we obtain  $\lambda = 194(16)$  nm and  $\kappa = 2.6(2)$  ( $f = 0.43$ ). Similar values have been obtained by μSR measurements (G. M. Luke, personal communication), and our value of  $\kappa$  is also supported by estimates of the critical field  $H_c$  from recent heat-capacity measurements on high-quality samples (S. Nishizaki and Y.M., unpublished data). The intensity of the weak  $\{1, 1\}$  spots is difficult to separate from that of the  $\{1, 0\}$  reflections, but appears to be somewhat larger than that given by the GL model with these parameters. We could not observe  $\{2, 0\}$  reflections, and this is in accord with calculations of their expected intensity. It should be remembered that GL theory is only exact close to  $T_c$  and the calculations used a single-component order parameter, which may not be the case here. However, the mutual consistency of these results encourages us to believe that we have obtained reliable superconducting parameters for our sample.

We now compare our value of  $B_{c2}(T = 0)$  with estimates from the Fermi surface properties, using the predictions of the BCS theory. With a cylindrical Fermi surface<sup>22</sup> for band  $i$  of radius  $k_F^i$  with carriers of effective mass  $m = m_i^* m_e$ , where  $m_e$  is the electron mass:

$$B_{c2}(0) = \frac{2\pi}{\gamma} \Phi_0 \left( \frac{k_B T_c m_e}{\hbar^2} \right)^2 \left( \frac{m_i^*}{k_F^i} \right)^2 \quad (2)$$

Here  $\ln(\gamma)$  is Euler's constant (0.577); we note that this equation is slightly different from the standard BCS result, which is for a spherical Fermi surface. We have included a band index  $i$  as the Fermi surface in SRO consists of three bands. As suggested elsewhere<sup>23</sup>, the value of  $B_{c2}(0)$  in a multi-band superconductor will be controlled by the band giving the highest value. The calculated values are shown in Table 1, and it is clear that the  $\gamma$ -band is in surprisingly good agreement with our observations. These values strongly suggest that at least in high fields, the super-



**Figure 2** Observations in the  $B$ - $T$  plane of a square FLL. By neutron scattering, a square FLL was observed at points marked with a square; at those marked with a cross, there was insufficient intensity to detect a FLL. The temperature dependence of  $B_{c2}$  for our sample with field parallel to **c** is also shown (filled circles). The transition was determined by measurement of the in-phase response of the a.c. susceptibility,  $\chi'$ ; a typical trace (at  $T = 70$  mK) is shown as the inset.

**Table 1 Fermi surface properties and superconducting parameters of  $\text{Sr}_2\text{RuO}_4$**

|                                       | $\alpha$ (holes)                         | $\beta$ (electrons) | $\gamma$ (electrons) |
|---------------------------------------|------------------------------------------|---------------------|----------------------|
| $k_F$ ( $\text{\AA}^{-1}$ ) (ref. 10) | 0.302(2)*                                | 0.621(3)            | 0.750(4)             |
| $N_i$ (ref. 10)                       | 0.216(2)                                 | 0.914(9)            | 1.334(13)            |
| $m_i^*$                               | 3.4(2)                                   | 7.5(4)              | 14.6(7)              |
| $m_i^*/k_F$                           | 11.3(6)                                  | 12.1(6)             | 19.5(10)             |
| $N_i/m_i^*$                           | 0.064(3)                                 | 0.122(6)            | 0.091(5)             |
| $B_{c2}(0)$ (mT) Eq. (2)              | 19(2)                                    | 22(2)               | 58(6)                |
| $\lambda_L(0)$ (nm) Eq. (3)           | $\alpha + \beta + \gamma: 98(2)^\dagger$ |                     |                      |
| $\lambda_L(0)$ (nm)                   | $\alpha + \beta: 120(3)$                 |                     | $\gamma: 171(4)$     |
| $\lambda_L$ (experiment)              | 194(16) nm: from SANS at 100 mK          |                     |                      |
| $B_{c2}(0)$ (experiment)              | 63 mT: from a.c. measurements            |                     |                      |

Values of  $m_i^*$  are revised values from ref. 13.

\* Error in last figure in parentheses.

† Value using all three bands and  $V_M = 5.71 \times 10^{-6} \text{ m}^3$  (ref. 32).

conductivity of SRO is mainly due to the  $\gamma$ -band, because one would expect the other bands to be depaired at these fields. (However, it should be noted that more exotic pairing could alter the numerical factors in equation (2).)

We can also calculate the low-temperature value of the penetration depth  $\lambda(0)$  from the Fermi surface properties, and compare this with  $\lambda$  from the SANS results. The free-electron expression is:  $1/\lambda^2(0) = ne^2\mu_0/m_e$ , where  $n$  is the electron density. If there are several bands, this generalizes (for either spherical or cylindrical Fermi surfaces) to:

$$\frac{1}{\lambda^2(0)} = \frac{e^2\mu_0}{m_e} \sum_i \frac{n_i}{m_i^*} = \frac{N_A e^2\mu_0}{V_M m_e} \sum_i \frac{N_i}{m_i^*} \quad (3)$$

where  $N_i$  is the number of electrons per formula unit in band  $i$  and  $V_M/N_A$  is the volume of one formula unit. Table 1 shows the result of applying equation (3) to all three bands: the value of  $\lambda$  comes out much smaller than our low-temperature experimental value. Now  $\lambda$  represents the strength of Meissner screening at long distances, and it depends only on the response of the electronic charges to the magnetic vector potential  $A$ , and not on the nature of the pairing. It can be shown<sup>24</sup> that all parts of the Fermi surface that have a gap contribute to the low-temperature value of  $\lambda$ . It seems clear that reasonable agreement with our other results can only be obtained by assuming that under the conditions of our experiment only the  $\gamma$ -surface contributes significantly to  $\lambda$ .

All our results are consistent with ideas of orbital-dependent  $p$ -wave superconductivity<sup>8,9</sup>. Within that framework, a non-time-reversal-invariant superconducting state is expected<sup>9</sup>, and has recently been observed<sup>25</sup>. We briefly discuss alternative schemes. One is a “non-unitary” pairing state<sup>26,27</sup>, which also breaks time-reversal symmetry and can have nodes in the gap. However, such a state only occurs (as seen in the A1 phase of  $^3\text{He}$ ) if some extra stabilizing factor such as strong coupling is present<sup>26</sup>. Estimates of the coupling strength<sup>28</sup> make this state unlikely. We should also consider  $d$ -wave pairing: however, to break time-reversal symmetry all the way to  $T_c$ , one needs two degenerate states, and the only candidate ( $\Gamma_2^+$  in the nomenclature of table IV in ref. 29) has nodes on the Fermi surface, where the  $z$ -component of the wavevector  $k_z$  is zero. Such a  $k_z$ -dependence of the gap is not expected in a two-dimensional material such as SRO. We conclude that the symmetry and orientation of the FLL and the values of the superconducting parameters all indicate that superconductivity in strontium ruthenate occurs primarily on the  $\gamma$ -sheet of the Fermi surface, and that the  $p$ -wave model<sup>8,9</sup> gives the most consistent explanation of our results as a whole. □

## Methods

**Sample preparation and properties.** The sample of  $\text{Sr}_2\text{RuO}_4$  was grown by the floating-zone technique with excess  $\text{RuO}_2$  as a flux<sup>30</sup>. It formed a rod of

approximately elliptical cross-section ( $2.5 \times 3.5 \text{ mm}$ ), with the  $c$ -direction of the tetragonal structure (perpendicular to the  $\text{RuO}_2$  planes) along the short axis of the ellipse, and one of the  $a/b$  directions  $\sim 30^\circ$  from the axis of the rod.  $T_c$  was 1.28 K (mid-point) with width (10–90%) of  $\sim 60 \text{ mK}$ , measured by low-frequency a.c. susceptibility. The highest  $T_c$  so far obtained in this material is 1.48 K, so our sample was subject to a small ( $\sim 15\%$ ) depairing by the residual electron scattering.

**SANS techniques.** A length of 8 mm was diamond-cut from the growth rod; this was held mechanically, and by a small quantity of glue, to a copper plate, mounted on the mixing chamber of a dilution refrigerator. This was placed between the poles of an electromagnet with holes parallel to the field for transmission of neutrons. The magnetic field was parallel to the  $c$ -axis of the crystal within  $0.5^\circ$ , and the FLL was observed using long wavelength neutrons, incident nearly parallel to the applied field, on instrument D22 at the Institut Laue Langevin. Typical wavelengths employed were  $14 \text{ \AA}$ , with a spread (full-width at half-maximum) of 12%. Transmitted neutrons were registered at a  $128 \times 128$  pixel multidetector (pixel size  $7.5 \times 7.5 \text{ mm}$ )  $17.71 \text{ m}$  beyond the sample. The main beam was intercepted by a Cd beamstop and the weak diffracted beams due to the FLL were extracted from the background scattering from sample and cryostat by subtracting data taken above  $T_c$ .

**Measurement of integrated intensity.** Integrated intensities of FLL diffraction spots lying in the horizontal plane could be measured by rotating the electromagnet and the sample together about a vertical axis, rocking these spots through the Bragg condition. The integrated intensity  $I_{hk}$  of a  $(h, k)$  reflection with wavevector  $q_{hk}$  may be related to a Fourier component  $F_{hk}$  of the magnetic field variation inside the FLL, via the relationship:  $I_{hk} = 2\pi\phi(\mu/4)^2(V\lambda_n^2/\Phi_0^2q_{hk}^2)|F_{hk}|^2$ , where  $\phi$  is the incident flux of neutrons of wavelength  $\lambda_n$ ,  $V$  is the sample volume,  $\Phi_0$  is the flux quantum and  $\mu$  is the neutron magnetic moment in nuclear magnetons. In initial measurements, we formed the FLL by cooling the sample in constant applied field. This gave a rather broad rocking-curve width of several degrees, which reduced the peak intensity and made the integration for  $I_{hk}$  inaccurate. We suspect that the width was due to random pinning and bending of the flux lines into a non-optimum configuration (the large anisotropy of SRO will make the flux lines particularly easy to bend). In  $\text{NbSe}_2$ , another anisotropic superconductor, the quality of the FLL under such circumstances was improved by passing a current<sup>31</sup>. We therefore induced currents in our sample by oscillating the applied field by  $\pm 1 \text{ mT}$  at  $\sim 0.5 \text{ Hz}$  during cooling. This increased the peak diffracted intensity by a factor  $\sim 2$  and the rocking-curve width was reduced to  $\sim 1.5^\circ$  half-width at half-maximum (the integrated intensity should be unchanged). The diffraction pattern shown in Fig. 1 was observed after cooling in this manner. The rocking-curve width was still large compared with the Bragg  $\theta$  for all the diffracted spots, so the complete pattern is seen with the incident neutron beam parallel to the field. It will be noted that the vertical spots are  $\sim 1.6$  times more intense than the horizontal spots: we believe that this is because with a (nearly vertical) rod-shaped sample, most residual flux-line bending will be in the horizontal direction.

**Measurement of  $B_{c2}$ .** This was determined from the a.c. susceptibility (0.2 mT at 97 Hz applied parallel to  $c$ ) of a small piece cut from the growth rod immediately adjacent to the SANS sample, as a function of temperature and magnetic field  $B$  applied parallel to the crystal  $c$ -axis. A typical in-phase response as a function of magnetic field (swept up or down) at constant temperature is shown in Fig. 2 inset. At low levels of excitation the flux lines are pinned, so a strong diamagnetic response is seen as soon as the sample enters the mixed state from the normal state.  $B_{c2}$  is taken as the mid-point of the a.c. transition.

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## Surface-promoted replication and exponential amplification of DNA analogues

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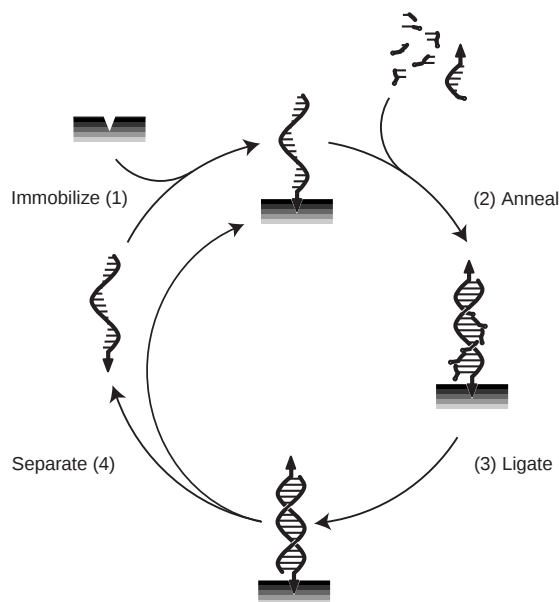
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Self-replicating chemical systems have been designed and studied to identify the minimal requirements for molecular replication<sup>1</sup>, to translate the principle into synthetic supramolecular systems<sup>2</sup> and to derive a better understanding of the scope and limitations of self-organization processes<sup>3</sup> that are believed to be relevant to the origin of life on Earth<sup>4</sup>. Current implementations make use of oligonucleotide analogues<sup>5–12</sup>, peptides<sup>13–17</sup>, and other molecules<sup>18–24</sup> as templates and are based either on autocatalytic, cross-catalytic, or collectively catalytic pathways for template formation. A common problem of these systems is product inhibition, leading to parabolic instead of exponential amplification<sup>25</sup>. The latter is the dynamic prerequisite for selection in the darwinian sense<sup>26,27</sup>. We here describe an iterative, stepwise procedure for chemical replication which permits an exponential increase in the concentration of oligonucleotide analogues. The procedure employs the surface of a solid support and is called SPREAD (surface-promoted replication and exponential amplification of DNA analogues).

Copies are synthesized from precursor fragments by chemical ligation on immobilized templates, and then liberated and immobilized to become new templates. The process is repeated iteratively. The role of the support is to separate complementary templates which would form stable duplexes in solution. SPREAD combines the advantages of solid-phase chemistry with chemical replication, and can be further developed for the non-enzymatic and enzymatic amplification of RNA, peptides and other templates as well as for studies of *in vitro* evolution and competition in artificial chemical systems. Similar processes may also have played a role in the origin of life on Earth, because the earliest replication systems may have proliferated by spreading on mineral surfaces<sup>28–33</sup>.

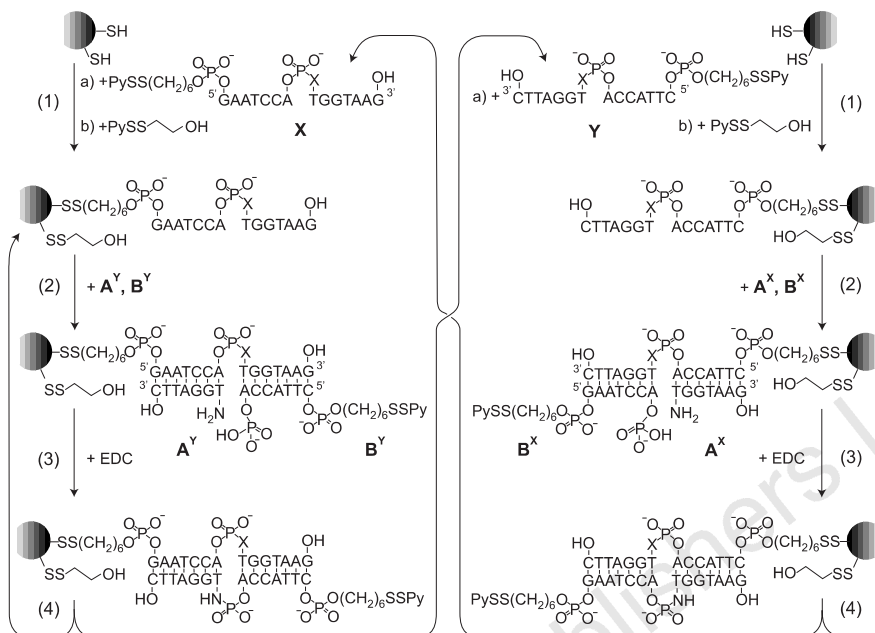
Stepwise 'feeding' procedures were previously employed in two different chemical systems that were reported as models of potentially prebiotic processes<sup>10,34,35</sup>. Li and Nicolaou achieved chemical replication of duplex DNA composed of palindromic (symmetrical) homopyrimidine and homopurine strands<sup>10</sup>. The homopyrimidine strand was synthesized from its precursor fragments via triple helix ligation, and then served as a template for the chemical ligation of the precursors of the homopurine strand. Thus, stepwise feeding with homopyrimidine and homopurine fragments prevented fragment complexation and therefore allowed switching between the respective triplex and duplex ligation intermediates. Ferris *et al.* have demonstrated the synthesis of long oligonucleotide- and peptide-like materials on the surface of mineral supports<sup>34,35</sup>. In these systems, stepwise feeding enabled the replenishment of activated precursors, and thus overcame the length-limiting effect of precursor hydrolysis. The conjunction of the above approaches, stepwise chemical replication and solid-phase chemistry, forms the basis of our procedure (Fig. 1).

For a demonstration of SPREAD (Fig. 2), two complementary 14-meric templates, X and Y, as well as four template fragments, A<sup>x</sup>, B<sup>x</sup>, A<sup>y</sup> and B<sup>y</sup>, were synthesized using standard phosphoramidite chemistry. A thiol-modified support was obtained from



**Figure 1** General scheme of the SPREAD procedure. (1) A template is immobilized by an irreversible reaction with the surface of a solid support. (2) The template binds complementary fragments from solution. (3) The fragments are linked together by chemical ligation. (4) The copy is released, and re-immobilized at another part of the solid support to become a template for the next cycle of steps. Irreversible immobilization of template molecules is thus a means to overcome product inhibition.





**Figure 2** Oligonucleotide analogues and reactions employed in the experiment. The individual steps of procedure (1)–(4) were performed separately for the complementary templates **X** and **Y**, with each template in a separate tube. PySS denotes a 2-pyridyl-disulphide moiety that is cleaved during immobilization (1a) and capping (1b) to give 2-thiopyridone. The backbone modification **X** at the central inter-nucleotide link of **X** and **Y** is **X** = N–H, except for the first immobilization where **X** = O. **A<sup>x</sup>**, **B<sup>x</sup>**, **A<sup>y</sup>** and **B<sup>y</sup>** denote the corresponding template frag-

ments. The hybridization step (2) gives rise to a termolecular complex from the immobilized templates and the respective fragments **A<sup>x</sup>**, **B<sup>x</sup>**, **A<sup>y</sup>** and **B<sup>y</sup>**. In the presence of the water-soluble carbodiimide EDC, chemical ligation (3) leads to a 3'–5' phosphoramidate linkage between an adjacent 5'-amino and a 3'-phosphate group. Each resulting double-stranded complex is denatured (4) yielding the templated support, as well as a PySS-modified copy that is immobilized on fresh SH support.

commercially available 2-pyridyldisulphide-activated agarose (Sephacrose 6B, Pharmacia) by reduction with dithiothreitol (DTT). Using disulphide exchange reactions<sup>36</sup>, the templates **X** and **Y** were separately immobilized on two batches of SH support to give the templated supports **X0** and **Y0**. The efficiency of the immobilization step was determined by the HPLC analysis of the supernatant (Fig. 3a, b). The remaining thiol groups of the supports were capped by reaction with S-(2-thiopyridyl)-2-mercaptoethanol. Fragments **A<sup>x</sup>**, **B<sup>x</sup>** and **A<sup>y</sup>**, **B<sup>y</sup>** were then hybridized on the immobilized templates, **Y0** and **X0**, respectively. To determine the hybridization efficiency a fraction of the support was reduced by DTT and analysed by HPLC (Fig. 3c). Chemical ligation<sup>37,38</sup> was achieved by replacing the hybridization buffer with a ligation buffer containing N-ethyl-N'-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC) as the condensing agent. To determine the efficiency of ligation, product formation was monitored by the HPLC analysis of the mixture of products obtained after cleaving the disulphide bonds of an aliquot of the templated supports using DTT as the reducing agent (Fig. 3d). The copies were then liberated by rinsing the supports with 0.1 M NaOH, analysed by HPLC, and re-immobilized on two new batches of the SH support to yield the templated supports **X1** (copy from the templated support **Y0**) and **Y1** (copy from the templated support **X0**). For the next generation, the whole cycle of steps was repeated with each of the four batches **X0**, **Y0**, **X1** and **Y1** to give **Y2**, **X2**, **Y3** and **X3**, respectively. An additional round generated the templated supports **X4**–**X7**, **Y4**–**Y7** (Fig. 3e, f).

Figure 4 summarizes the pathway of each individual support together with the amount of material obtained after each copying cycle. Generally, the yield of an replication cycle  $p$  need not necessarily reach  $p = 1$  in order to enable an exponential mode of amplification. For the case of palindromic templates, where **X** = **Y**, **A<sup>x</sup>** = **A<sup>y</sup>**, **B<sup>x</sup>** = **B<sup>y</sup>**, the amount of material  $x_n$  obtained from an

initial amount  $x_0$  after  $n$  replication cycles is given by:

$$x_n = x_0(1 + p)^n \quad (1)$$

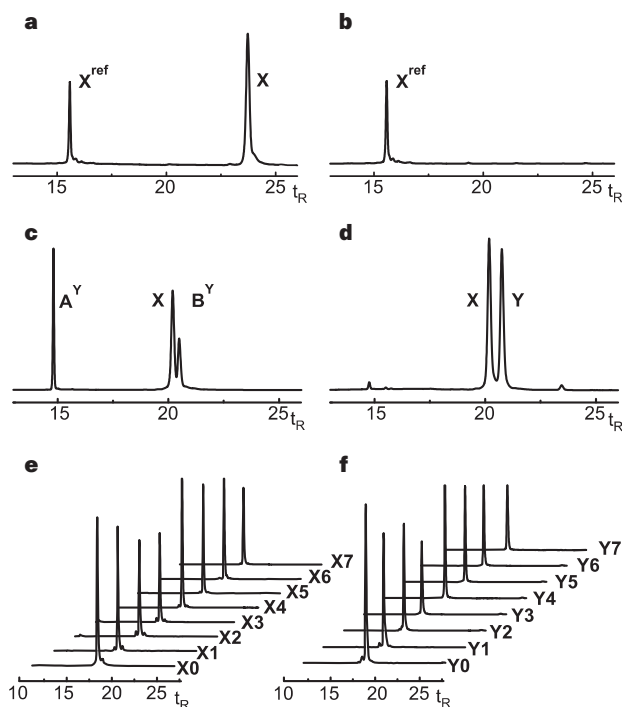
allowing an exponential increase for the condition  $p > 0$ . For the case of non-palindromic templates underlying our experiments, each round of replication consists of two copying cycles with the individual yields  $p_x$  and  $p_y$  for the synthesis of **X** and **Y**, respectively. Here, the amount of immobilized templates **X** and **Y** follows the iterative progression:

$$x_{n+1} = x_n + p_x y_n \quad \text{and} \quad y_{n+1} = y_n + p_y x_n \quad (2a, b)$$

requiring both  $p_x > 0$  and  $p_y > 0$  for an exponential increase. Equation (2a, b) gives;

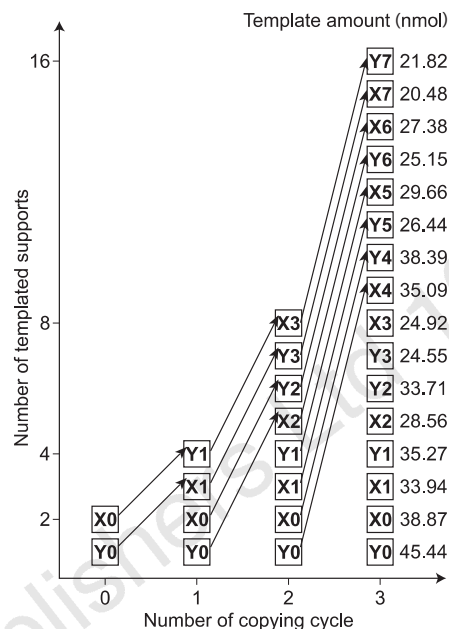
$$\begin{aligned} x_n &= \frac{1}{2} \left( x_0 + y_0 \sqrt{\frac{p_x}{p_y}} \right) \left( 1 + \sqrt{p_x p_y} \right)^n \\ &+ \frac{1}{2} \left( x_0 - y_0 \sqrt{\frac{p_x}{p_y}} \right) \left( 1 - \sqrt{p_x p_y} \right)^n \\ y_n &= \frac{1}{2} \left( y_0 + x_0 \sqrt{\frac{p_y}{p_x}} \right) \left( 1 + \sqrt{p_x p_y} \right)^n \\ &+ \frac{1}{2} \left( y_0 - x_0 \sqrt{\frac{p_y}{p_x}} \right) \left( 1 - \sqrt{p_x p_y} \right)^n \end{aligned} \quad (3a, b)$$

where  $x_0$  and  $y_0$  denote the initial amount of **X** and **Y**, respectively. A comparison of experimental and theoretical values for  $x_n$  and  $y_n$ , as given in Fig. 4 legend, confirms that the data shown in Fig. 4 are consistent with an exponential mode of amplification for the template materials.



**Figure 3** HPLC analysis of products under denaturing conditions obtained in the consecutive steps of a SPREAD cycle. All HPLC samples without reduction buffer (RB) were brought to a concentration of 100 mM DTT before analysis (except **a**) to ensure reproducibility of HPLC quantification.  $t_R$  is the retention time in minutes. **a**, Reaction mixture containing **X** and GAATCCATGGTAAG (**X<sup>ref</sup>**) as internal standard before immobilization. **b**, Supernatant after immobilization. **c**, Reaction mixture after hybridization of immobilized **X** with **A<sup>y</sup>** and **B<sup>y</sup>** and treatment of the support with RB. **d**, Reaction mixture after chemical ligation and reductive cleavage from the support using RB. **e, f**, HPLC analysis of the set of 16 samples obtained from the reductive cleavage of immobilized templates that were generated by three SPREAD cycles. For the symbols to the right of the HPLC profiles, see Fig. 4. We note that the small amounts of impurities visible in the front profiles are consecutively removed in the course of SPREAD amplification.

The SPREAD procedure demonstrates exponential amplification in chemical replication. Solid-phase chemistry is a prerequisite for a practicable automation of the procedure, as basically pipetting and filtration steps are involved. Exponential amplification opens the opportunity for directed molecular evolution: in this context, the procedure has the potential for controllable mutation frequency, as copy liberation in step (4) (see Fig. 1) should depend on the thermodynamic stability of the immobilized duplexes and thus mutants should elute faster than perfect copies. Protocols based on the cycling of temperature (or other environmental parameters) as well as on solution chemistry are perhaps less applicable for chemical systems based on short templates. Fast negative temperature jumps as well as a fast condensation chemistry were needed to prevent the re-equilibration of the oligonucleotide complexes involved. These conditions would then necessarily limit the fidelity of template copying, because the thermodynamic control of molecular recognition sets the threshold for copying fidelity in the absence of an energy-dissipating 'proof-reading' mechanism. We thus conclude that SPREAD may be of considerable value for the design of evolutionary chemical systems. Other templates, surfaces, and template-surface links may be employed, enzymatic variants may be explored, and different steps may be combined to arrive at a higher level of process integration and autonomy. Protocols are conceivable in which related templates are immobilized close to each other, thus giving the concept of quasispecies<sup>3</sup> a spatial



**Figure 4** Pathway of template transfers in the course of three cycles of SPREAD amplification. Boxes and lettering symbolize reaction tubes and templated supports, respectively. Arrows indicate which copy is generated from which template. The amount of template material shown was quantified by HPLC analysis after cleavage of the disulphide links by reduction with DTT. From these data, 14 individual yields were calculated. The average yields and standard errors are:  $p_x = 0.763 \pm 0.071$  and  $p_y = 0.855 \pm 0.079$ . The number of nanomoles of each template at cycle  $n$ , compared with its theoretical value (in brackets) as calculated from equation (3a, b), is as follows:  $x_0 = 38.87$  (38.87),  $y_0 = 45.44$  (45.44),  $x_1 = 72.81$  (73.54),  $y_1 = 80.71$  (78.68),  $x_2 = 126.29$  (133.58),  $y_2 = 138.97$  (141.57),  $x_3 = 238.90$  (241.61),  $y_3 = 249.77$  (255.80).

dimension. Furthermore, protocols in which two or more different classes of template molecules—such as oligonucleotides and peptides—are involved may contribute to the selection and discovery of more complex, cooperative systems. In the short term, the procedure is ready to be applied to the replication of peptides<sup>13</sup>, pRNA<sup>11</sup>, and enantiomeric forms of RNA<sup>39</sup>. In the long run, the search for autonomous variants of SPREAD may lead to a self-sustaining chemical system capable of undergoing darwinian evolution, compatible with Joyce's definition of life<sup>40</sup>. □

#### Methods

**Materials.** The synthesis of **X**, **Y**, **A<sup>x</sup>**, **A<sup>y</sup>** and **B<sup>y</sup>** will be reported elsewhere. Reduction buffer (RB): 100 mM DTT, 40 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, pH 7.9; immobilization buffer (IB): 370 mM sodium acetate/acetic acid, 0.5 M NaCl, 1 mM EDTA, pH 4.4, degassed with argon for oxygen liberation; capping buffer (CB): 60 mM *S*-(2-thiopyridyl)-2-mercaptoethanol, 40 mM MES, 0.5 M NaCl, 1 mM EDTA, pH 6.5; hybridization buffer (HB): 100 mM MES, 40 mM MgCl<sub>2</sub>, 1 M NaCl, 1 mM 2,2'-dipyridyldisulphide, pH 6.1; ligation buffer (LB): HB with 0.2 M EDC, fresh before chemical ligation.

**General methods.** Unless otherwise specified, all supported reactions were performed at 25 °C in Mikro-Spin centrifuge filters (0.5 ml, cellulose acetate with 0.45-μm pores, Roth) stacked into Eppendorf tubes (1.5 ml). Each of a series of 16 filters was loaded with ~50 mg of wet Sepharose 6B (washed according to the manufacturers' guidelines) and then centrifuged (3 min at 4,000g) to remove the supernatant. Before an immobilization step, the beads in

400  $\mu$ l RB were vortexed for 1 h to generate the thiol form of Sepharose 6B. RB was then replaced by IB, repeating buffer addition, sonification (10 s), and centrifugation six times.

**Immobilization.** A suspension of SH Sepharose in 400  $\mu$ l IB containing 20–40 nmol of the respective template was agitated by vortexing at 1,000 r.p.m. for 1 h.

**Capping.** 100  $\mu$ l CB was added to the above suspension. After agitation for 1 h, the beads were filtered by centrifugation, resuspended in 400  $\mu$ l CB and vortexed for 1 h at 25 °C. CB was then replaced by a solution of 200 mM S-(2-thiopyridyl)-2-mercaptoethanol in ethanol. After vortexing for 2 h at 70 °C, the beads were washed with ethanol (5 $\times$  with 200  $\mu$ l) and 1 M NaCl (5 $\times$  with 200  $\mu$ l).

**Hybridization.** The beads were resuspended in 250  $\mu$ l HB containing the complementary heptamers at 400  $\mu$ M concentration. The temperature was raised to 85 °C, decreased to 4 °C within 1 h, and kept there for 2 h. Excess heptamers were removed by washing the beads with three 200- $\mu$ l portions of 1 M NaCl at 4 °C.

**Chemical ligation.** The beads were resuspended in 375  $\mu$ l LB, vortexed for 40 h at 4 °C, and washed with 200  $\mu$ l 1 M NaCl at 4 °C three times.

**Denaturation.** A Mikro-Spin filter with the respective beads was inserted into an Eppendorf tube containing 150  $\mu$ l 1 M acetate buffer (pH 4.4). The beads were resuspended and gently agitated in 50  $\mu$ l 0.1 M NaOH, and the supernatant was transferred by centrifugation (7,000g, 30 s) into an Eppendorf tube. After repeating this procedure three times, the resulting solution (400  $\mu$ l) was ready for the next cycle. The beads were recycled by washing with four portions of HB.

**HPLC.** All HPLC samples without reduction buffer (RB) were brought to a concentration of 100 mM DTT before analysis to ensure reproducibility. All separation were performed on a RP C-18 column (250/4, Nucleosil 120-5 AB, Macherey and Nagel). Eluates: 0.1 M triethylammonium acetate (pH 7)/MeCN 1% (A) and MeCN (B). Analytical measurements were taken at 50 °C by using a gradient of 2% to 6% A in 2 min, 6% to 25% A in 30 min, and a flow rate of 1 ml min<sup>-1</sup>. The eluate was monitored simultaneously at 254 nm and 273 nm. Equipment (Kontron): two pumps 422, autosampler 465, diode array detector 440, Kroma 2000 was used as data acquisition system.

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## Iron acquisition by photosynthetic marine phytoplankton from ingested bacteria

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Iron is unique among biologically essential trace metals in having a higher particulate than dissolved concentration in ocean surface waters<sup>1</sup>. Uptake of dissolved iron is generally considered to be the norm for phytoplankton, as even the smallest iron-bearing particles are unavailable for transport into cells<sup>2,3</sup>. But the oceanic dissolved fraction is so small, and the particulate fraction so inert<sup>2</sup>, that phytoplankton production is limited by a dearth of available iron in some regions<sup>4</sup>. Here we use incubation experiments to show that *Ochromonas* sp., a common photosynthetic flagellate from the Pacific Ocean, can obtain iron directly in particulate form, by ingesting bacteria. Iron acquisition is highly efficient; *Ochromonas* assimilates 30% of the ingested ration, acquiring a high intracellular iron concentration and maintaining a significantly faster growth rate than when iron is provided in the dissolved phase. Phytoplankton capable of such phagotrophy (so-called mixotrophic species) may thus be able to assimilate iron in both particulate and dissolved forms in the ocean. Moreover, when iron availability is limited, the iron 'cost' of growth is diminished because *Ochromonas* derives a greater fraction of its energy from the bacteria. Analysis of standing



**Table 1 Elemental composition and metabolic properties of *Ochromonas* sp.**

| Fe source*               | mol Fe per cell<br>( $10^{-18}$ ) | mol C per cell<br>( $10^{-12}$ ) | Fe:C<br>( $\mu\text{mol mol}^{-1}$ ) | $\mu$ †<br>( $\text{d}^{-1}$ ) | Fe use efficiency<br>(mol C per mol Fe $\text{d}^{-1}$ ) | C from bacteria<br>(%) | GFeGE‡<br>(%) | FeRe§<br>(%) |
|--------------------------|-----------------------------------|----------------------------------|--------------------------------------|--------------------------------|----------------------------------------------------------|------------------------|---------------|--------------|
| Dissolved: Fe-sufficient | 187 ± 58                          | 1.26 ± 0.4                       | 149 <sup>a</sup> ± 46                | 1.28 <sup>a</sup> ± 0.13       | —                                                        | —                      | —             | —            |
| Bacterial: Fe-rich       | 117 ± 30                          | 0.765 ± 0.16                     | 154 <sup>a</sup> ± 40                | 1.37 <sup>a</sup> ± 0.09       | —                                                        | 12–7.9                 | 36.5 ± 5      | 65 ± 3       |
| Dissolved: Fe-deficient  | 32.8 ± 8.9                        | 1.23 ± 0.27                      | 26.6 <sup>b</sup> ± 6                | 0.71 <sup>c</sup> ± 0.13       | $2.67 \times 10^{ab} \pm 0.54 \times 10^4$               | —                      | —             | —            |
| Bacterial: Fe-poor       | 18.9 ± 1.1                        | 0.786 ± 0.3                      | 24.0 <sup>b</sup> ± 1                | 1.07 <sup>b</sup> ± 0.1        | $4.45 \times 10^{ab} \pm 0.25 \times 10^4$               | 48–43.5                | 28.1 ± 1.2    | 66 ± 9       |

Superscripted letters denote significant differences among the treatments using a Tukey test in a 1-way ANOVA ( $p < 0.0001$ ).

\* Fe was supplied to *Ochromonas* cultures as dissolved Fe (dissolved:Fe-sufficient, 8.41  $\mu\text{M}$ ; dissolved:Fe-deficient, 12.9 nM) or as particulate Fe bound in bacteria (bacterial:Fe-rich, 36 ± 27 nM; bacterial:Fe-poor, 3.6 ± 0.9 nM).

† Specific growth rate.

‡ Gross Fe growth efficiency.

§ Fe regeneration efficiency.

stocks and clearance rates of plankton in the equatorial Pacific shows that the iron flux through mixotrophic flagellates can amount to 35–58% of the total Fe uptake by the entire autotrophic community. Our results suggest that the phagotrophic ingestion of bacteria may be an effective adaptive strategy for photosynthetic organisms to obtain iron for growth in iron-limited regions of the sea.

Eukaryotic phytoplankton are thought to take up Fe solely through a saturable transport system in which Fe binds to a surface receptor and is subsequently internalized across the cell membrane<sup>5</sup>. The concentration of labile Fe(III) species and their rates of water loss control the kinetics of the reaction. At oceanic levels, diffusion limits Fe uptake as labile species account for only 0.03% of the total dissolved pool of 70 pM (ref. 1). The remaining dissolved Fe is bound to strong organic complexes<sup>1</sup> that are not directly available for phytoplankton transport. Some species may be able to utilize this complexed Fe by reduction<sup>6</sup>. Alternative mechanisms of Fe acquisition by eukaryotic phytoplankton are at present unknown<sup>3</sup>.

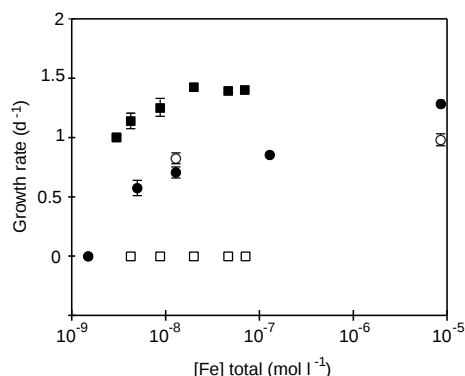
*Ochromonas* sp. was grown in artificial seawater media with high and low concentrations of dissolved Fe. Like other members of the Chrysophyceae<sup>7,8</sup>, and many Prymnesiophyceae, this species is a mixotroph that can phagocytose bacteria and fix carbon photosynthetically. The *Ochromonas* species that we examined had to photosynthesize as it was unable to grow in complete darkness even when provided with  $>10^7$  bacteria  $\text{ml}^{-1}$  as a C source. Maximum division rates were achieved at high [Fe], but steadily declined as total [Fe] was reduced to 1.5 nM whereupon no growth was observed (Fig. 1). When the flagellate was supplied with bacteria as the only source of Fe, significantly faster rates of growth were attained at all low Fe concentrations. The maximum rates were

similar to those observed with high concentrations of dissolved Fe, demonstrating that the primary stimulatory factor provided by bacteria was Fe. Dissolved Fe concentrations in the bacteria-Fe treatments were apparently negligible because *Thalassiosira pseudonana*, a diatom that acquires Fe from solution<sup>3</sup>, was unable to grow (Fig. 1). Final cell densities of the cultures were dependent on the nitrogen concentration in the medium, and were higher in the presence of bacteria as *Ochromonas* assimilated some bacterial N as previously observed for C, N and P in other mixotrophs<sup>7–9</sup>.

Differences in bacteria density ( $(0.1\text{--}5) \times 10^7$  cells  $\text{ml}^{-1}$ ) did not greatly influence the rate of algal growth ( $1.25\text{--}1.4$   $\text{d}^{-1}$ ), although changes in bacteria Fe content did. When the total Fe concentration of bacteria was  $<5$  nM, *Ochromonas* was Fe-limited and grew at ~70% of its maximum rate (Fig. 1). The bacteria prey were themselves Fe-deficient, but had a C content (10 fmol C per cell) and C:N ratio similar to the Fe-rich bacteria<sup>10</sup>. As algal biomass increased exponentially during growth, bacteria densities declined by 1–2 orders of magnitude. Ingestion rates were significantly faster in the Fe-limited than sufficient cultures,  $14 \pm 1.4$  versus  $9 \pm 0.9$  bacteria per mixotroph  $\text{h}^{-1}$ , as a result of greater clearance rates ( $2 \pm 0.3$  versus  $0.4 \pm 0.05$  nl per mixotroph  $\text{h}^{-1}$ ). Thus, Fe deficiency appears to induce a physiological response in the mixotrophs that increases acquisition of the limiting resource.

To quantify transfer of Fe from the ingested bacteria, we labelled them with <sup>55</sup>Fe and monitored incorporation of the <sup>55</sup>Fe into the phagotrophic phytoflagellates. At high and low concentrations of total Fe, ~30% of the bacterial Fe was assimilated, similar to that observed with heterotrophic flagellates<sup>11</sup> and metazoan grazers<sup>12</sup>. The remainder was regenerated and released back into solution. In excess of 90% of the egested Fe passed through 0.2- $\mu\text{m}$  filters, indicating that it was dissolved or bound to very small particles. Iron content (Fe quota:  $\mu\text{mol Fe per mol C}$ ) of *Ochromonas* decreased fivefold under Fe-deficiency as expected, but was not affected by the Fe source (dissolved versus particulate) at the concentrations we tested (Table 1). Growth rates, however, were significantly faster in the Fe-limited cultures amended with bacteria. This result was surprising, because phytoplankton growth is well known to be proportional to cellular Fe quota at low Fe (ref. 13) and hence should be independent of the type of Fe assimilated if quotas are the same.

The faster rates were apparently achieved by the flagellates adopting a more heterotrophic mode of existence, as ~45% of their C was obtained from ingested bacteria (Table 1). Bacterial C could be derived by one or both the following pathways. Some of it may represent the incorporation of pre-formed organic compounds for biosynthesis or alternatively, some of it may be catabolized during oxidative metabolism to generate ATP and subsequently fixed as CO<sub>2</sub> by the photosynthetic reactions. The latter pathway seems more likely as 65% of the regenerated bacterial C was CO<sub>2</sub> regardless of whether the flagellates were grown on high or low Fe bacteria. The increased assimilation of bacterial C under Fe-limiting conditions is most likely to have resulted from indirect C incorporation. Both processes should be Fe economical and are predicted to decrease the Fe costs for C assimilation compared to pure photoautotrophic growth<sup>14</sup>.



**Figure 1** Utilization of dissolved and particulate Fe by *Ochromonas*. Growth rates of *Ochromonas* sp. in bacterial (filled squares) and dissolved (filled circles) iron media as a function of the total concentration of Fe in the media. Growth of *Thalassiosira pseudonana* (clone Fer) in bacterial (open squares) and dissolved (open circles) iron media was used as a control. Clone Fer, which was derived from *Thalassiosira pseudonana* (3H) (M. T. Maldonado and N. M. Price, unpublished results), maintains near-maximum rates of growth at low concentrations of dissolved Fe.

**Table 2 Biogenic Fe flux in the equatorial Pacific Ocean**

| Plankton group                 | Abundance (l <sup>-1</sup> )      | Clearance rate (nl per organism d <sup>-1</sup> ) | Primary production rate (nmol C l <sup>-1</sup> d <sup>-1</sup> ) | Fe flux (pmol Fe l <sup>-1</sup> d <sup>-1</sup> ) | Assimilated Fe (pmol Fe l <sup>-1</sup> d <sup>-1</sup> ) | Regenerated Fe (pmol Fe l <sup>-1</sup> d <sup>-1</sup> ) |
|--------------------------------|-----------------------------------|---------------------------------------------------|-------------------------------------------------------------------|----------------------------------------------------|-----------------------------------------------------------|-----------------------------------------------------------|
| Mixotrophic nanoflagellates    | 5 × 10 <sup>5</sup> (Refs 20, 22) | 240 (Refs 22, 27)                                 | –                                                                 | 2.7*                                               | 0.81†                                                     | 1.76                                                      |
| Mixotrophic dinoflagellates    | 1 × 10 <sup>6</sup> (Ref. 20)§    | 310 (Ref. 28)                                     | –                                                                 | 0.7*                                               | 0.21†                                                     | 0.48                                                      |
| Heterotrophic nanoflagellates  | 7 × 10 <sup>5</sup> (Ref. 20)     | 240 (Refs 22, 27)                                 | NA                                                                | 3.8*                                               | 1.14†                                                     | 2.66                                                      |
| Total photosynthetic community | –                                 | NA                                                | 700–1,138 (Ref. 29)                                               | 5.8–9.5‡                                           | 5.8–9.5                                                   | NA                                                        |

NA, not applicable.

\* Estimated by multiplying the total amount of Fe bound in heterotrophic bacteria, cyanobacteria and prochlorophytes (22.5 pmol Fe l<sup>-1</sup>, refs 10, 20, 30) by grazer abundance and clearance rate.

† Estimated using a GFeGE of 30% (this study and ref. 11).

‡ Estimated using the Fe quota of 9 μmol Fe: mol C (pico, nano and microplankton: ref. 10) for regenerated production and 3.6 μmol Fe: mol C for new production (ref. 3), and an *f*-ratio of 0.12 (ref. 31).

§ Estimated assuming 10% of pigmented cells are mixotrophic.

|| Estimated using an ReFeE of 65% for mixotrophic species (this study), and a FeReE of 70% for heterotrophic species (ref. 11).

We quantified the Fe costs for C assimilation by calculating iron-use efficiencies (IUE)<sup>14</sup> for *Ochromonas* under Fe-limitation (Table 1). Large differences in IUE are evident between coastal and oceanic phytoplankton, and are thought to have resulted from evolutionary adaptation to the Fe levels in the environment<sup>13</sup>. The photosynthetic IUE reported here is half that observed in other coastal autotrophs<sup>15</sup>, possibly reflecting the ability of *Ochromonas* to acquire Fe from the particulate pool. When bacteria were supplied as the Fe source, the phytoflagellates assimilated almost twice as much C per unit Fe. Thus, energy acquisition derived via bacterial grazing increases the Fe economy of the cell and minimizes rate limitation in this photosynthetic flagellate.

The observation that a photosynthetic flagellate can obtain Fe by phagotrophy adds a new dimension to our understanding of Fe acquisition by phytoplankton, and may be relevant to the ecology of natural waters. Particulate Fe is composed primarily of colloids, detritus, bacteria and cyanobacteria<sup>3</sup> and represents the largest reservoir of Fe in the surface ocean. Low concentrations of dissolved Fe severely limit growth of diatoms and other large algal species that bloom following Fe enrichment<sup>4</sup>. Phagotrophic phytoflagellates may be able to fulfil their Fe requirements by directly utilizing some of the particulate Fe pool and at the same time to reduce the amount of Fe needed for growth by being more heterotrophic. These abilities may explain in part their lack of response during Fe fertilization experiments<sup>4</sup> and the reported high *in situ* growth rates of indigenous phytoplankton communities in Fe-limited parts of the sea<sup>3</sup>.

As *Ochromonas* excreted some of the Fe it ingested, phagotrophic phytoflagellates may in general play an important role in the Fe cycle by regenerating Fe for themselves and for other primary producers. In oceanic regions, regeneration is the most important process providing Fe for phytoplankton growth<sup>12</sup>. Heterotrophic organisms are considered to be solely responsible for this supply<sup>11,12,16</sup>, although zinc and toxic-metal regeneration has been demonstrated previously by a freshwater ochromonad feeding on cyanobacteria<sup>17</sup>. The simultaneous release and consumption of Fe represents a 'short circuit' in the microbial loop in that the grazer and the producer are the same organism. We speculate that mixotrophic phytoplankton might also be able to utilize dissolved, organically-bound Fe via saprotrophy<sup>9,18</sup> or the detrital and colloidal pools that are thought to be even more abundant<sup>3</sup>.

Chrysophyceae, Prymnesiophyceae and Dinophyceae are abundant and ubiquitous in the world's oceans<sup>18,19</sup> and generally dominate the eukaryotic biomass of Fe-limited regions<sup>3,19–21</sup>. They contain large numbers of species that are known for their ability to consume particles<sup>9,18</sup>. Studies during JGOFS cruises to the equatorial Pacific showed that similar proportions of the phototrophic and heterotrophic nanoflagellates were ingesting picoplankton<sup>22</sup>, and that at least 27–54% of micro- and mesoprotozooplankton<sup>23</sup> were simultaneously both photosynthetic and predatory. Thus, the mixotrophic nutritional strategy may be

associated with an important fraction of the biogenic iron standing stock as well as its flux. Our calculations show that Fe uptake by consumption of picoplankton by mixotrophs accounts for 35–58% of the Fe utilized by the entire autotrophic community (Table 2). Although variations in the degree to which mixotrophic species acquire C from ingested particles and from solution will undoubtedly influence their contribution to autotrophic production, their Fe flux is significant in terms of Fe acquisition and regeneration. If the gross Fe growth efficiency of mixotrophs is 30%, then assimilation of Fe by this pathway amounts to 11–17% of the Fe used each day by all photosynthetic organisms in the equatorial Pacific (Table 2). The unassimilated Fe regenerated by mixotrophs is 24–39% of that required by the primary producers. As the chemical reactivity of the regenerated Fe varies<sup>11</sup>, and because bacteria compete with phytoplankton for this resource<sup>10</sup>, the proportion of it that is re-assimilated by the primary producers is unknown. We note, however, that similar amounts of Fe are regenerated by mixotrophic and heterotrophic nanoflagellates. These values, however, should be regarded as minimum estimates as we have not considered the possible uptake and regeneration of Fe from the more abundant detrital and colloidal pools.

Despite the practical and conceptual difficulties presented by simultaneous heterotrophy and photosynthesis in microorganisms, studies of elemental cycling should now consider the significant contribution of mixotrophy. In particular, the ability of photosynthetic organisms to exploit smaller competitors and to influence biogeochemical cycles by acquiring and regenerating Fe from particulate forms may be of great importance in Fe-limited regions of the sea. □

## Methods

**Growth and grazing rates of *Ochromonas*.** *Ochromonas* sp. was obtained from the North East Pacific Culture collection (NEPCC no. 457). Growth rates were determined by measuring *in vivo* fluorescence of cultures once or twice daily using a Turner Designs 10-AU fluorometer. The phytoflagellates were grown in acid-washed 30-ml polycarbonate tubes<sup>24</sup> at 20 °C under continuous saturating light (200 μE m<sup>-2</sup> s<sup>-1</sup>). Specific growth rates were determined from linear regression of the log<sub>e</sub> *in vivo* fluorescence versus time during the initial exponential growth phase. *Ochromonas* cultures were not axenic and initially contained ~5 × 10<sup>4</sup> bacteria ml<sup>-1</sup>.

For grazing rates, changes in flagellate and bacterial abundance were monitored over time by staining filtered samples with DAPI and counting cells using epifluorescence microscopy. Duplicate cultures (400 ml) containing iron-rich and iron-poor bacteria were inoculated with 500–1,000 mixotrophic cells ml<sup>-1</sup>. Abundance changes were followed for 8–10 d. Grazing rates were calculated by dividing the change in bacterial abundance by the total flagellate abundance (obtained by integration of the growth curve) during the growth phase.

**Determination of Fe quotas.** Cells were grown in AQUIL<sup>24</sup> containing 50 μM NH<sub>4</sub><sup>+</sup> as the nitrogen source. Iron was added to the medium in a dissolved form as an Fe(III) solution complexed to EDTA or as Fe contained in living

bacteria. Two concentrations of dissolved Fe were added: iron-sufficient ( $[\text{total Fe}] = 8.41 \mu\text{M}$ , computed concentration of inorganic  $\text{Fe(III)}$ ,  $[\text{Fe}'] = 40 \text{ nM}$ , although ferric hydroxides will precipitate at this concentration), and iron-deficient ( $[\text{total Fe}] = 12.9 \text{ nM}$ ,  $[\text{Fe}'] = 25 \text{ pM}$ ). Quotas were measured using  $^{55}\text{FeCl}_3$  ( $0.52 \text{ Ci mol}^{-1}$ , DuPont) which was added as 1% and 100% of total Fe in the sufficient and deficient media, respectively<sup>15</sup>. Sodium  $^{14}\text{C}$ -bicarbonate ( $5 \text{ Ci mol}^{-1}$ , Dupont) was added at 1% of the final concentration to all dissolved media.

A marine bacterium, strain Ju188<sup>10</sup>, isolated from the Sargasso Sea was used as a particulate source of Fe. Cultures were grown to stationary phase in 400 ml of Fe-sufficient AQUIL with  $50 \text{ mg l}^{-1}$  glucose, or in iron-deficient AQUIL with  $100 \text{ mg l}^{-1}$  glucose<sup>10,11</sup>. Iron was added as  $^{55}\text{Fe}$  as described above.  $^{14}\text{C}$ -glucose ( $3 \text{ Ci mol}^{-1}$ , Amersham) was 1% of the final glucose concentration. To remove dissolved Fe, the bacteria were centrifuged, washed twice in Fe-free AQUIL with  $100 \mu\text{M}$  EDTA and the final bacterial pellet was resuspended in Fe-free AQUIL. The mean quotas of the iron-rich and iron-poor bacterial prey were  $101 \pm 32$  and  $3.92 \pm 1 \mu\text{mol Fe per mol C}$ , respectively.

Mixotrophs grown in these different media were harvested at late exponential phase, filtered onto  $2\text{-}\mu\text{m}$  polycarbonate filters and rinsed with  $\text{Ti(III)}$ -citrate EDTA reagent to remove extracellular iron<sup>25</sup>. Bacteria trapped on these filters represented  $<1\%$  of the total bacterial biomass. Bacterial abundance and particulate iron concentration in control flasks without flagellates did not change significantly during the experiments.

$^{14}\text{C}$  and  $^{55}\text{Fe}$  activity was measured by dual-label liquid scintillation counting. Iron quotas were measured in 3 separate experiments, each of which had between 2 and 4 replicates. Samples used for quota determinations were fixed simultaneously for biomass determinations by epifluorescence microscopy. Quotas for cells grown on bacteria were derived by dividing the amount of Fe:cell determined using radiotracers by the mean C:cell from image analysis.

**Determination of *Ochromonas* C content.** Photographs were taken of DAPI stained *Ochromonas* slide preparations using a camera mounted on a Leica epifluorescence microscope ( $400\times$ ). Photos were scanned and binarized to size the cells using UTHSCSA Image Tool version 1.27. At least 25 cells were sized per treatment. Standard error of estimated volumes was 5–8%. Cell carbon was estimated using a factor of  $150 \text{ fg C } \mu\text{m}^{-3}$  (ref. 26).

Cell C biomass estimates derived using image analysis (IA) were compared with those estimated using the  $^{14}\text{C}$  incorporated by the mixotrophs and dividing by cell abundance in the dissolved treatments in order to verify the choice of the C conversion factor. Mean C estimates among methods were not significantly different (Fe-sufficient dissolved:  $\text{IA} = 1.26 \pm 0.4 \text{ pmol C}$  vs  $^{14}\text{C} = 1.4 \pm 0.22 \text{ pmol C}$ ,  $p = 0.26$ , and Fe-deficient dissolved:  $\text{IA} = 1.23 \pm 0.27 \text{ pmol C}$  vs  $^{14}\text{C} = 1.21 \pm 0.2 \text{ pmol C}$ ,  $p = 0.81$ ).

**Regeneration and gross-growth efficiencies.** For dissolved activity ( $^{55}\text{Fe}$ ), samples from the quota experiments were filtered through a  $0.2\text{-}\mu\text{m}$  Acrodisc syringe-tip filter. Regeneration efficiencies were calculated from the total amount of Fe measured in the dissolved phase divided by the total amount of Fe ingested<sup>11</sup>. Gross Fe growth efficiencies were calculated by dividing the total  $^{55}\text{Fe}$  activity in the mixotrophs at the end of a given time interval by the total  $^{55}\text{Fe}$  activity ingested during the same interval, determined from the total number of bacteria grazed multiplied by the  $^{55}\text{Fe}$  per bacterium (ref. 11). As we did not control for the possibility that regenerated Fe was reassimilated by *Ochromonas*, the Fe regeneration efficiencies and the gross Fe growth efficiencies should be regarded as maximum and minimum estimates, respectively. Carbon derived by heterotrophic growth was calculated by dividing the amount of bacterial C retained by *Ochromonas* when grazing on  $^{14}\text{C}$ -labelled bacteria by the total amount of C per *Ochromonas* determined by image analysis. The  $^{14}\text{C}$  contained in undigested bacteria (between 1 and 4 bacteria per cell) was subtracted from the total  $^{14}\text{C}$  measured in the mixotrophs.

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## Seismic evidence that the source of the Iceland hotspot lies at the core–mantle boundary

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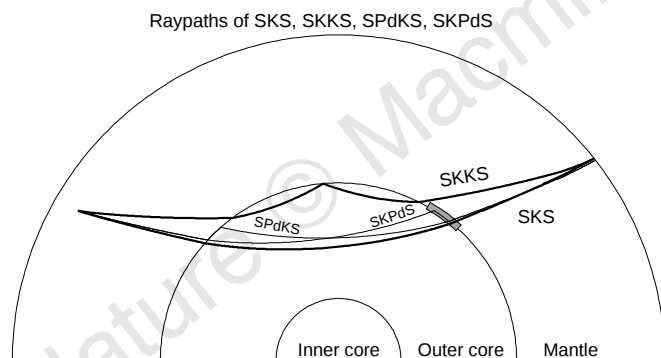
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Although Morgan<sup>1</sup> proposed in 1971 that hotspots such as Iceland were the result of hot, rising mantle plumes, it is still debated whether plumes originate from a thermal boundary just above the core–mantle boundary or at the base of the upper mantle<sup>2</sup>. Although seismic evidence of plumes in the upper mantle is accumulating<sup>3</sup>, narrow plume conduits in the deep mantle have yet to be detected. Details of plume formation in the lower mantle have therefore remained largely unconstrained<sup>4</sup>. Here, however, we present seismic evidence for the presence of a localized patch of material with ultra-low seismic wave speed, located at the core–



mantle boundary beneath the Iceland hotspot, and propose that this zone represents the hot, partially molten source region of the Iceland mantle plume. Through the modelling of seismic waveforms, we constrain the seismic velocity structure at this patch of the core–mantle boundary using a numerical–analytical interfacing code<sup>5</sup> designed to reproduce the complex interference of shear-wave phases transmitted through, and refracted at, the boundary<sup>6</sup>. Although this structure is difficult to constrain precisely, our preferred model consists of a dome which is 250 km wide, 40 km high and contains P- and S-wave velocity (wave-speed) reductions of 10% and 30%, respectively.

Recent advances in seismological research have shown that the seismic phase SKS and associated P diffractions SPdKS and SKPdS are extremely useful for the study of structure at the base of the mantle. SPdKS and SKPdS differ from SKS by two short segments of P-wave paths at the base of the mantle, while their propagation paths in the upper and middle mantle are virtually identical<sup>7</sup> (Fig. 1). The complex waveform interference of these phases are strongly sensitive to P and S velocity structure at their core–entry and core–exit points<sup>8</sup>. Most observations of anomalous interference between SKS and SPdKS (or SKPdS) have been observed for core–mantle boundary (CMB) sampling regions which lie beneath the central Pacific where strongly varying P-wave velocity reductions of up to 10% have been found. These structures appear to be centred beneath a broad low-shear-velocity region of the lower mantle constrained by anomalously delayed S-SKS and SKS-SKKS differential travel times<sup>9,10</sup>. Normal SPdKS observations are found for regions sampled around the Pacific Ocean and can be modelled by standard seismic velocity models such as PREM<sup>11,12</sup>. These features can be



**Figure 1** Ray-path geometry. Cross-section through the PREM (preliminary reference Earth model)<sup>25</sup> showing the ray-paths of SKS, SKKS and two diffracted phases SPdKS (source-side) and SKPdS (receiver-side) at a distance of 118°. The latter phases are caused by the critical angle S-to-P and P-to-S conversion at the CMB which produces P-wave diffractions along this boundary. PREM-like models, with constant velocities across D° (the transition zone at the CMB), predict the bifurcation of SKS near 106° which leads to a long-period waveform interference near 112°, depending on the source duration. If the P-wave velocity is reduced, the interference shifts to shorter distances. Identifying this bifurcation point, therefore, becomes an excellent tool for fixing the P-wave velocity at a particular location on the CMB. We note that the only significant region sampled differentially by these paths occurs in the 1-D outer core and at the CMB. The paths through the crust and mantle are essentially identical<sup>7</sup>. This minimizes the uncertainties of waveform modelling due to crust and mantle heterogeneities, and source radiation pattern. The box indicates a localized region where the velocity structure can be complex<sup>5</sup>. Following a hybrid approach, we compute the velocities and stresses on the leading edge of the box with a generalized ray code assuming a line source. The motions are propagated across this zone containing the ULVZ with the aid of a finite-difference technique. The motions are then interfaced with an analytic code after applying the Kirchhoff method and propagated to the receivers. Detailed comparisons of the output of this hybrid method against analytical methods for plane-layered Earth models demonstrates its accuracy at the various stages of interfacing<sup>5</sup>.

**Table 1** South American events list

| No. | Origin          | Latitude (deg) | Longitude (deg) | Depth (km) |
|-----|-----------------|----------------|-----------------|------------|
| 1   | 700604 04:09:25 | −9.9           | −78.9           | 57         |
| 2   | 790521 22:22:23 | −15.44         | −70.04          | 209        |
| 3   | 700617 4:44:20  | −16.0          | −71.88          | 99         |
| 4   | 731025 14:8:58  | −21.96         | −63.65          | 517        |
| 5   | 680823 22:36:49 | −21.95         | −63.64          | 513        |
| 6   | 671227 8:53:51  | −21.2          | −68.3           | 135        |
| 7   | 690725 6:6:42   | −25.49         | −63.21          | 573        |
| 8   | 831212 12:21:12 | −28.13         | −63.15          | 602        |
| 9   | 670909 10:6:44  | −27.62         | −63.15          | 577        |

seen in Fig. 2b, where SKS remains relatively simple out to 111° and the separation between SKKS and SKS is easily modelled by PREM-like models. This simplicity is in contrast to observations obtained at KEV and KRK from South American events (Fig. 2c). The enhanced P-diffractions can be produced by decreasing the P-velocity in a radially uniform model (1-D) which shifts the S-to-P critical angle to smaller distances at each CMB crossing<sup>7,14</sup> and produces the secondary pulse. These segments are indicated in map-view in Fig. 2a for great-circle paths. There is some evidence for broadening on the VAL and STU records (Fig. 2a) and an associated increase in SKKS-SKS differential timing separation indicative of lower velocities<sup>11</sup>; however, these CMB samples (Fig. 2a) are located considerably south of the KEV and KRK samples.

Here we analyse recordings of more South American events (Table 1) to further resolve the CMB structure causing the waveform complexity evident in Fig. 2c. These data are displayed in Fig. 3 and grouped according to whether SKS/SPdKS/SKPdS waveform interference is anomalous or normal (PREM-like). The anomalous observations are primarily observed at stations KEV, KRK and KBS, where the separation of SPdKS from SKS develops much earlier than for the normal observations. Complex earthquake rupture characteristics can be ruled out as the cause of the waveform because waveforms at NUR and UME for the same earthquake are relatively simple. Moreover, we infer from this waveform variation at the European stations that SKPdS, but not SPdKS, is the anomalous phase, as the source-side CMB segments for all stations are closely located (Fig. 2a). Additionally, SPdKS waveforms sampling beneath the Americas are normal<sup>8</sup>, that is, as displayed in Fig. 2b.

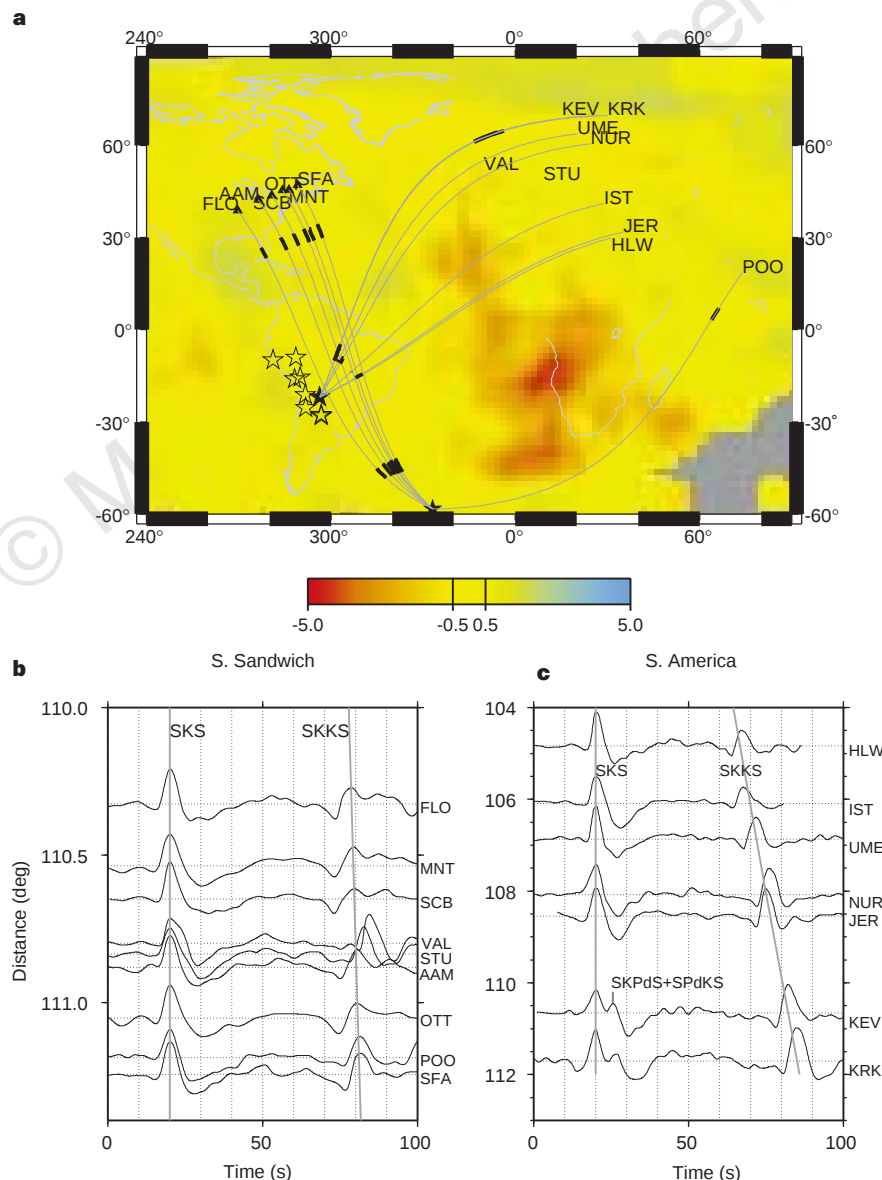
The great-circle paths from the nine events sampling beneath the North Atlantic are displayed in Fig. 4. Again the diffraction paths are indicated by the heavy lines with SKKS and SKS exit points. As SKS and SKKS arrivals fit PREM (Fig. 2c), we can restrict the anomalous region to the area within the heavy contour enclosing the KEV and KRK segments. Henceforth, we will presume that the anomalous waveforms displayed in Fig. 3 are caused by the structure labelled ULVZ (ultra-low-velocity zone).

Considerable progress has been made in modelling anomalous waveforms of the type displayed in Fig. 3 using laterally varying lower-mantle seismic-velocity models<sup>14</sup>. Modelling of SPdKS and SKPdS has been conducted using horizontally stratified velocity layers that are different at the core entry and exit points of the two diffractions. They then arrive at different times, which reduces their overall interference. Fortunately, the branch containing the ULVZ becomes large and occurs earlier in distance. If the ULVZ is at the exit point, the upcoming P wave from the core crosses the zone as P and can be reflected back down to the CMB where it changes mode to S, essentially SKPPS. As the internal reflection at the top of the zone can reach critical angle caused by the velocity increase, the phase SKPPS can be large over a limited distance range, controlled by the ULVZ P-velocity. Increasing this reflection by introducing local sloping structures produces synthetics fitting the KEV records<sup>15</sup>. Thus, we are driven to curve boundaries that can

effectively focus energy downwards, which is one of the reasons for developing the hybrid code discussed in Fig. 1 legend.

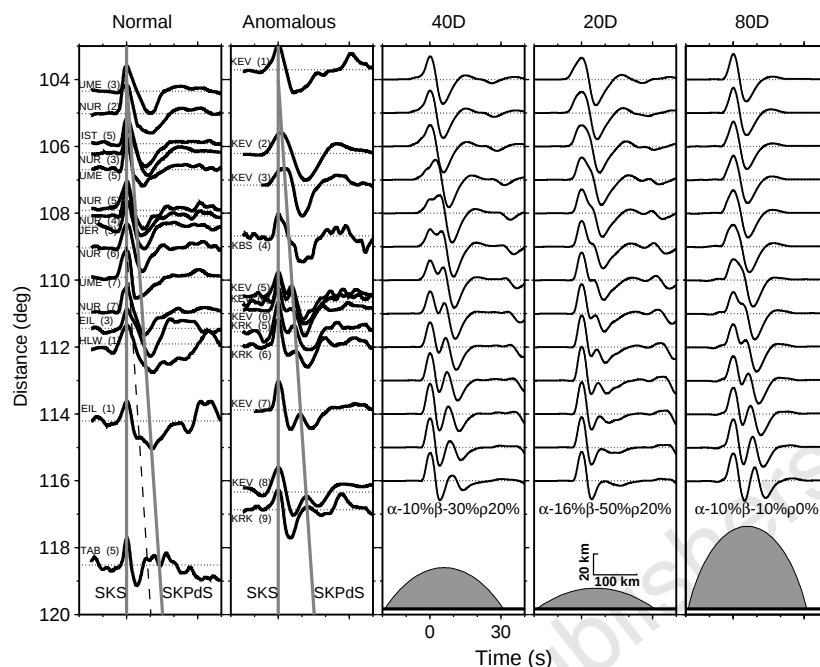
After experimenting with a variety of structural shapes (boxcars, domes and triangles), we found that dome shapes produce the types of waveforms typically observed from ULVZs<sup>5</sup>. To sample the model space, we conducted sensitivity tests with various widths, heights and S-velocities. The trade-off of density with S-wave velocity has been discussed previously<sup>8,16</sup>. Structures with widths less than 100 km produce no discernible effects on long-period synthetics, irrespective of their position at the CMB. Broad structures with widths greater than 500 km produce results looking more like the one-dimensional layered synthetics but with reduced SKPPS amplitudes<sup>5</sup>. Dome widths of ~250 km produce the strongest interference with SKS, when the dome is centred on the Pd segment. This shape fits nicely into the contour lines of the ULVZ displayed in Fig. 4 and provides the fortunate set of circumstances allowing this

detailed modelling. We selected three sets of synthetics to convey these results (Fig. 3). Synthetics for model 40D are considered to provide the overall best fit as determined by waveform matching (overlay). The 20D model with the reduced shear velocity of 50% fits the top set of records at distances of 106° to 109° the best, but the KBS record (109°) may not be sampling the same structure (see Fig. 4). The recording NUR (105°) is not anomalous but comes from the same event, producing KEV at 106° to demonstrate that the broadness at KEV is not caused by the source. Zones with thicknesses less than 20 km, with large S-wave velocity drops, are particularly good at producing broad pulses. Thicker zones generally produce a stronger SKPdS phase and the prominent waveform distortions. The 80D model with the S-wave velocity drop of 10% produces too much ringing at the larger distances. Thus, our preferred models have greatly reduced shear velocities, indicative of melt.



**Figure 2** Sampling of the core-mantle boundary and corresponding record section. **a**, Map showing events beneath South Sandwich Island (event 3 in Table 1) and central South America (event 5), along with great-circle paths and heavy line segments indicating the locations of  $P_{diff}$  paths (SPdKS and SKPdS) along with the velocity structure of the deep mantle<sup>26</sup>. Colour scale is +5% (blue) to -5% (red). **b**, **c**, Record sections of long-period WWSSN (World Wide Seismo-

graphic Station Network) data. The original analogue recordings have been digitized and rotated into SV (radial) and SH (tangential). The SV records have been aligned on the SKS phase and plotted as a function of epicentral distance in degrees. The dotted reference lines are those predicted by PREM indicating normal (SKKS-SKS) differential times and PREM-like lower mantle structures along these paths.

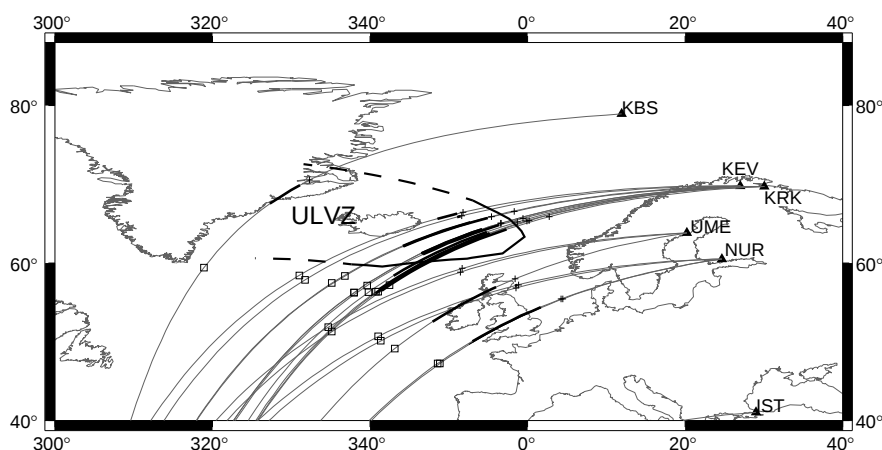


**Figure 3** Observations of South American events recorded in Europe divided into two groups, normal and anomalous, along with three sets of synthetics. The observations are aligned on SKS with lines indicating the approximate positions of the diffracted phase SKPdS. The station identification and location are given in Fig. 4 with event indexing keyed to Table 1. We note the complexity of a typical event recorded at KEV relative to NUR. The observations in this record section have been slightly shifted in distance ( $0.1^\circ$  change in distance for 50 km change in depth) to correct for the apparent shift in interference pattern<sup>8</sup>. The synthetics

were generated from models containing 2D dome structures with a horizontal scale of 250 km located within the patch (ULVZ) in Fig. 4 along the heavy SKPdS line segments. The dome height varies from 20 to 80 km with corresponding S-wave drops of 50 to 10% assuming a 10% drop in P-wave velocity for all models fixed by the apparent position of the interference line (SKPdS). These synthetics were generated with a WKB-type code<sup>13</sup> and contain a WWSSN long-period response, a correction for attenuation  $t^* = 1$ , and a trapezoidal source function (in seconds) (2,2,2) to be compatible with the observed data<sup>7</sup>.

To substantiate our interpretation of this waveform data and to resolve finer detail will require the use of other phases. Core reflections prove very useful for these purposes, as demonstrated for the ULVZ beneath Fiji<sup>17,18</sup> and other ULVZs. Distortions in the precursors to the PKP wavefield also appear useful<sup>19</sup>. These wavefields can be easily added to our hybrid method<sup>20</sup> but prove relatively insensitive to shear velocity variations, as in the case of PcP precursor<sup>17</sup>. Thus the direct seismic evidence presented here for a

greatly reduced shear velocity is probably the strongest case for partial melting, which is in agreement with estimates of the reduction of P-to-S velocity ratios based on the physics of molten aggregates<sup>21,22</sup>. Moreover, the relationship of this ULVZ with the upper-mantle thermal anomaly beneath Iceland<sup>23</sup> and the positions of ULVZs and hotspots<sup>24</sup> on a more global scale suggests that these structural features may be related to the “convecting plumes” proposed by the originators of plate-tectonic theory<sup>1</sup>. □



**Figure 4** Magnified view of the region beneath the North Atlantic indicating the core-exit points of SKS (crosses) and SKKS (squares). The heavy line segments indicate the diffracted paths along the CMB. The SKPdS segments associated with KEV and KRK appear to be sampling a localized ULVZ located beneath

Iceland. D'' velocity structure<sup>26</sup> beneath the North Atlantic is not particularly anomalous, in agreement with the normal travel times of SKS and SKKS at the European stations (Fig. 2c).



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## Seismic evidence for small-scale dynamics in the lowermost mantle at the root of the Hawaiian hotspot

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The hot thermal boundary layer produced by heat transport from the Earth's core to the base of the mantle is thought to contain strong horizontal shear flows and to nucleate instabilities in which hot material rises into the convecting mantle as thermal plumes<sup>1–3</sup>. A recent study<sup>4,5</sup> proposes that the Hawaiian plume is deflected by mantle convection and, in the lowermost mantle, is located to the southeast of its surface manifestation. Here we present seismic data that densely sample, with core-reflected shear

waves, a region beneath the central Pacific Ocean which includes the predicted location of the deflected root of the Hawaiian hotspot. Our mapping of the structure in this region of the lowermost mantle reveals strong lateral gradients in shear-wave velocity and anisotropic shear-wave polarization direction over distances of only several hundred kilometres. We interpret these gradients as being indicative of small-scale dynamical structure in the thermal boundary layer, where vertical flow into the Hawaiian plume at its root is accompanied by horizontal flow towards the plume.

We examine shear waves traversing the deep mantle below a region southeast of Hawaii from 54 earthquakes in the Tonga-Fiji region recorded on 34 digital broadband seismometers in western North America (Fig. 1a). This data set provides better spatial resolution than any other study of deep mantle anisotropy<sup>6</sup> because most previous studies used phases that diffract for long distances along the core-mantle boundary (CMB) and had less dense station distributions.

We analyse shear-wave reflections from the CMB at epicentral distances of 73° to 84° as these provide the best possible spatial sampling of deep mantle structure in our study area. Shallow mantle effects are suppressed by considering ScS differential times relative to the direct S phase (Fig. 1b), which turns at shallower depths in the mantle, and by applying corrections for models of anisotropic lithospheric structure beneath the receivers<sup>7,8</sup>. For the travel-time analysis we use the tangential components of motion (ScSH and SH), as these are free of complexities due to the core-traversing, longitudinally polarized SKS phase in our distance range. The ScS polarization on the tangential and longitudinal (ScSV) components is investigated in the shear-wave splitting analysis.

The central Pacific area studied is known to have anomalous shear-velocity structure in the lowermost mantle. Several global seismic tomography models indicate that our study area has slower-than-average shear velocity, in contrast to faster-than-average velocities characterizing regions beneath margins of the Pacific Ocean<sup>9–12</sup>. A shear-velocity model, M1, with a 3% velocity reduction in the lowermost 200–300 km of the mantle has been proposed for this region<sup>13</sup>, and there is compelling evidence for a layer (~10 km thick) above the CMB with strongly reduced velocities (5–10% decreases for P-wave velocity and 15–30% for S-wave velocity)<sup>14–17</sup>. The low shear velocities are likely to indicate hotter than average temperatures, with the very large velocity reductions at the base of the boundary layer suggesting partial melt<sup>18</sup>. Seismic anisotropy, manifested by shear-wave splitting, may result from mineralogical or structural alignments that reflect the strain and/or stress regime in a given region<sup>6,7,19</sup>. Anisotropy with SH faster than SV has been found to the northeast of our study area, and mixed or negligible anisotropy closer to our region<sup>20,21</sup>.

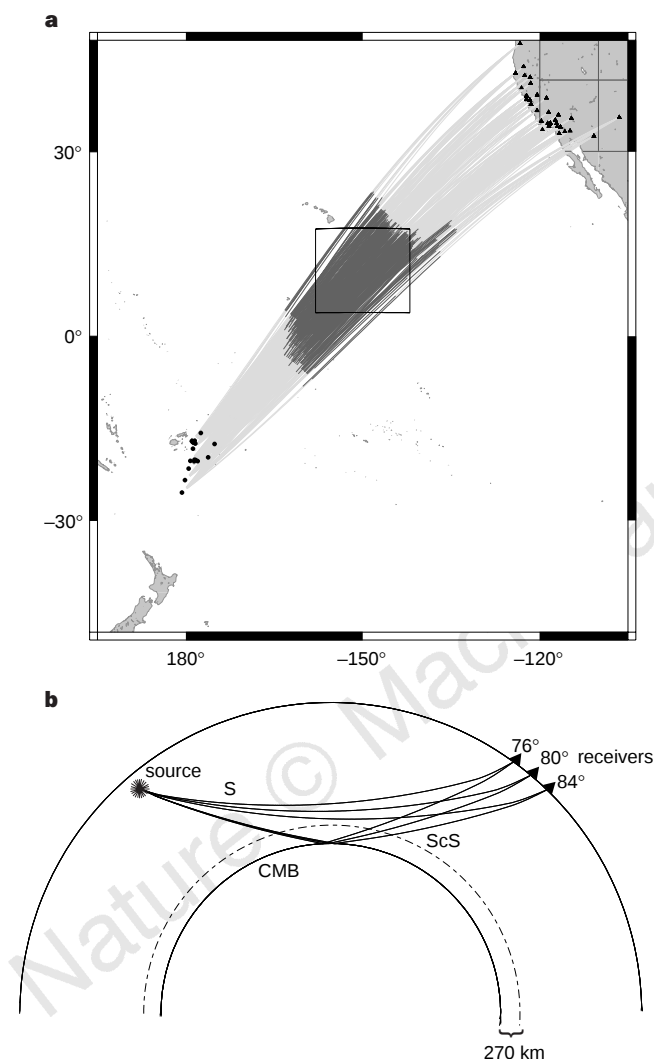
Our shear-wave signals are selected to be simple and impulsive to minimize the uncertainties involved in measuring arrival times. Using the cleanest arrival onsets and peaks, we measure differential times between ScSH and SH for a subset of 248 out of 762 seismograms. The differential travel times average 4 s larger than predicted by the reference velocity model PREM<sup>22</sup>, much of which can be accounted for by model M1<sup>13</sup>. We find a strong lateral gradient in the differential times anomalies increasing towards the northeast with respect to PREM (Fig. 2a). The ray-path geometry is such that this low-velocity structure need not be strictly confined to the base of the mantle, although absolute travel times indicate that ScS is the phase responsible for the differential behaviour. 3.5% lateral variations in shear velocity in the lowermost 250 km of the mantle over length scales of 600 km can account for the 4 s increase in ScS delay across our study region. Confining the anomalous zone to the lowermost 100 km of the mantle requires extreme lateral gradients of 7% over 300-km scale lengths. Conversely, distributing the anomalous structure over greater depth extent towards the northeast from the CMB reflection points in Fig. 2a allows reduction of

the magnitude of the velocity decrease. Although the geometry of the heterogeneous structure is not uniquely resolved, our data clearly sample a low-shear-velocity region with strong lateral gradients involving velocities that decrease in the northeasterly direction.

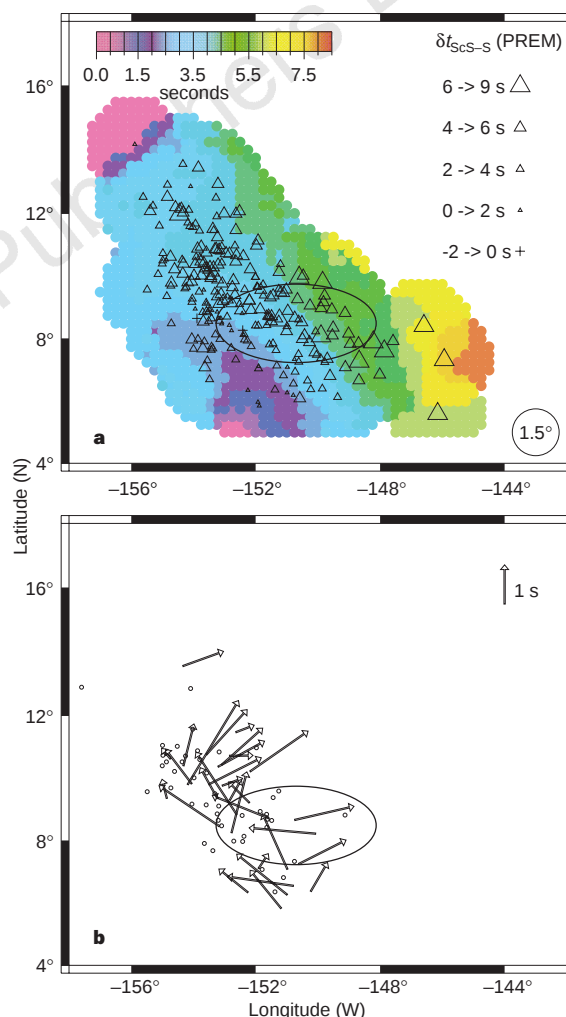
In addition, shear-wave splitting measurements were made for the 81 ScS observations that are most free of contamination from S and SKS arrivals. The splitting delays between the fast and slow polarizations are typically of the order of 1.0 s, with values as large as

2.0 s, after correction for lithospheric anisotropy and a small phase shift ( $\sim 0.2$  s at  $78^\circ$ ) of ScSV at the CMB. The directions of the fast polarization angles are plotted at the ScS reflection points in Fig. 2b. The data have significant scatter; however, estimates of the polarization direction are expected to be very unstable for small values of splitting delay (the lengths of the arrows in Fig. 2b are proportional to the splitting delay).

In the northeast portion of the study area, the larger splitting values are generally oriented in the along-path direction (from southwest to northeast), indicating that ScSV arrives earlier than ScSH. In the southwest, the larger splitting times are associated with fast directions in a nearly orthogonal direction (that is, ScSH travels faster than ScSV). This rotation of polarization is unlikely to be an artefact created by the lithospheric corrections as individual stations yield a wide range of splitting angles for rays that arrive at



**Figure 1** Geometry of ray-paths. Ray diagrams showing the ray-paths between the earthquake sources (magnitudes  $> 5.1$ , depths 250–650 km) in Tonga–Fiji and the digital broadband stations in the Berkeley Digital Seismic Network, Caltech USGS/TERRAScope and the IRIS (Incorporated Research Institutions for Seismology) seismic arrays. **a**, Surface projection of the ray-paths with the dark grey lines southeast of Hawaii indicating where ScS traverses the lowermost 270 km of the mantle. The box marks the region at the core-mantle boundary (CMB) encompassing the ScS reflection points. **b**, S and ScS ray-paths computed for model M113 for a source at a depth of 500 km at epicentral distances of  $74^\circ$ ,  $80^\circ$  and  $84^\circ$ . The direct S wave turns in the mid-mantle while ScS reflects off the CMB. The dominant periods of our observations are  $\sim 10$  s and the associated Fresnel zone at the CMB (the spatial extent sampled by the finite frequency reflection) is an ellipse elongated along the ray-path direction with principle axis dimensions of about  $5^\circ$  by  $10^\circ$ . The direct S phase is used as a reference to ScS to cancel common effects on their similar paths through the upper mantle. A comparison of absolute S and ScS travel-time anomalies with the ScS–S anomalies reveals that the ScS anomalies have a relatively stronger correlation. This indicates that ScS encounters anomalous low-velocity structure in the deep mantle where the ray-path separates from S.



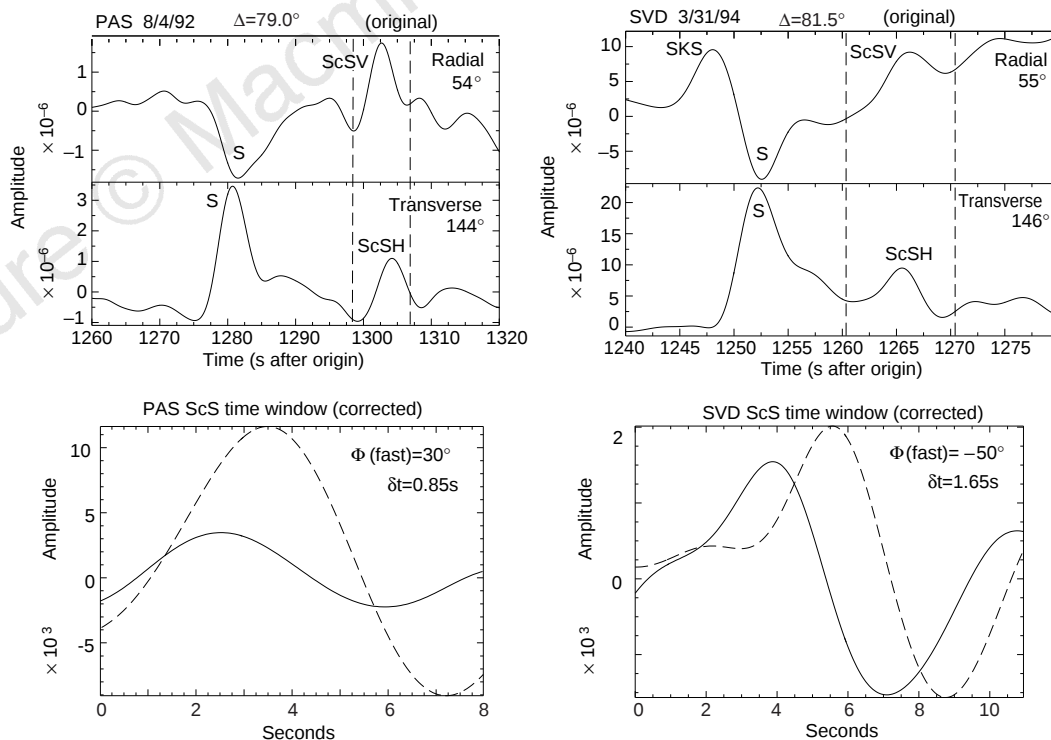
**Figure 2** Map views of the travel-time residuals and anisotropy measurements plotted at the ScS CMB reflection points. **a**, Travel-time residuals  $((\text{ScSH} - \text{SH})_{\text{obs}} - (\text{ScS} - \text{S})_{\text{PREM}})$  smoothed with a gaussian cap-average with cap radius of  $1.5^\circ$ . The colour scale is for the cap-average values. The ellipse represents the predicted location of the root of the Hawaiian plume based on mantle convection models. **b**, ScS fast polarization directions and splitting magnitudes plotted at their CMB reflections points. The arrows point in the direction of the fast component, as measured from north, and the length of the arrow indicates the magnitude of split. The circles represent data that either were too noisy to obtain a reliable measurement or did not possess any measurable splitting. There is a 0.4-s uncertainty in the splitting magnitudes and a  $23^\circ$  uncertainty in the polarization direction, based on the inverse  $F$  test<sup>28</sup>. A transition appears from fast directions perpendicular to the ray-paths in the southwest to fast directions parallel to the ray-paths in the northeast. The ellipse is the same as in **a**.

approximately the same incidence angle and with azimuths varying by less than  $7^\circ$ . There is no intrinsic requirement that the quasi-shear wave polarizations be related to the great-circle reference frame, but the few intermediate polarization directions found are mostly for observations with weak splitting. Figure 3 illustrates representative waveforms in the great-circle coordinate system, as well as the optimal fast/slow quasi-shear wave coordinate system, with the clear time offset of ScSV and ScSH almost coinciding with the optimal splitting.

This bimodal behaviour shows the simplest spatial pattern, segregating arrivals with different polarization directions, when the measurements are projected to the ScS turning points, shown in Fig. 2b. There is much more mingling of direction when the data are plotted at intersections with shallower depth surfaces. Shear-wave-splitting observations for direct S phases that traverse the deep mantle to the northeast of our study area show intermittent cases of fast SH or fast SV as well as no onset-time splitting<sup>21,23</sup>, suggesting that shear-wave splitting does not accumulate systematically with path length towards the northeast. These factors favour an interpretation of strong lateral variation in anisotropy on lateral scale lengths of 300–500 km within the lowermost mantle. Lateral gradients of 2–3% in the velocity ratio  $v_{SH}/v_{SV}$  and  $90^\circ$  changes in the fast polarization direction over scale lengths of 500 km, are required if we confine the variations to the thermal boundary layer at the CMB. As these are sub-Fresnel-zone scale lengths, it is difficult to constrain the actual structure, but Fig. 2 provides a strong case for coupled lateral gradients in shear velocity (with ScSH slowing relative to SH towards the northeast) and anisotropy (with ScSH slowing relative to ScSV towards the northeast). This gradient occurs near the postulated origin of the Hawaiian plume.

Although there are several viable mechanisms, sheared chemical heterogeneities or partial melt zones appear to be the leading candidates for producing seismic anisotropy near the base of the mantle<sup>6,24</sup>. Sheared melt inclusions can efficiently induce shear-wave anisotropy, even with small melt fractions<sup>25</sup>. In circum-Pacific regions, which contain higher than average shear velocities and anisotropy with  $v_{SH} > v_{SV}$ , several studies have suggested that down-welling oceanic slab structures, possibly involving partial melt or chemical heterogeneities within the slab, contribute to the anisotropy<sup>25,26</sup>. As our study area is far from zones of historic subduction, slabs are probably not involved. Given the presence of a very low velocity zone right at the base of the mantle that appears to involve partial melt<sup>18,27</sup>, and the overall low velocity structure in the region, the most likely explanation for our observations involves strong shear-flow-induced fabrics with inclusions of partial melt and/or chemical heterogeneity.

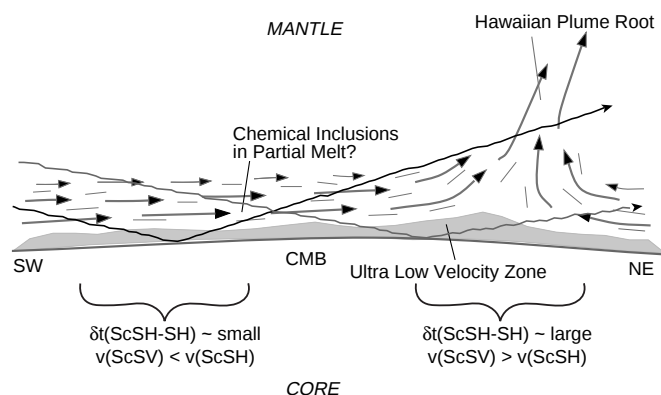
Lateral shear flows within the CMB boundary layer are likely to horizontally shear partial melt inclusions and to develop chemically distinct lamellae (Fig. 4). This can explain the observations in the southwestern portion of our study area of the relatively fast ScSH arrival (although still slow in an absolute sense). In the northeastern portion of the study area, a transition to vertical flow would create general anisotropy with vertical striations or lamellae of partial melt in the medium, allowing ScSV to travel faster than ScSH. It is likely that partial melt distributions are concentrated towards the CMB and, to the extent that this concentrates anisotropic structure towards the CMB, the magnitude of the anisotropy should increase with depth. Are such small-scale gradients common, as might be expected within a chaotic, unstable boundary layer, or are we seeing an extreme case due to the large magnitude of the expected



**Figure 3** Two seismograms from different events and stations which illustrate the presence of variable anisotropy across our study region. The upper panels show the uncorrected radial and transverse components of the S and ScS phases. The vertical lines denote the time window of the signals in the lower panel. The lower panels are the windowed corrected integrated ScS phases rotated to the fast/slow polarization direction  $\Phi$  (fast component shown by the solid line). Corrections for known lithospheric anisotropy were applied using parameters derived from S and SKS splitting measurements in previous studies<sup>7</sup>. The ScS angles of emergence are close to those of the SKS waves so the corrections are

appropriate. A small correction that varies with epicentral distance,  $\sim 0.2$  s, was also applied for the phase shift of ScSV on reflection from the CMB relative to ScSH. The corrected data were cross-correlated to seek a linear polarization of the ScS motion as a function of fast polarization angle and splitting delay<sup>7,8</sup>. A covariance method<sup>28</sup> to find the splitting parameters was also utilized and the values plotted are those obtained in both procedures. Standard particle motion and confidence region plots were also generated to confirm the reliability of these results<sup>28</sup>.





**Figure 4** A cartoon of the boundary layer at the CMB that incorporates the differential travel time and anisotropy observations. Differential shear flows that upwell into a mantle plume in the northeast affect ray paths differently depending on what region they sample. ScS reflecting in the southwestern region (left) would have ScSH travel faster than ScSV due to the horizontally sheared structure with embedded partial melt, while in the northeastern region (right) ScSV will travel faster than ScSH due to vertically sheared fabric of partial melt and chemical heterogeneity. The transition from lateral to vertical structure would impart different anisotropic splitting to the two ray-paths.

boundary layer inflow feeding the Hawaiian plume? The strong gradients in structure at the boundary layer revealed in our data support the presence of small-scale dynamics at the CMB. As this has many implications for heat transport and interactions between the core and mantle, further comparable high-resolution investigations of other regions are necessary to explore this dynamical regime. □

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## Sauropod dinosaur embryos from the Late Cretaceous of Patagonia

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Definitive non-avian dinosaur embryos, those contained inside fossil eggs, are rare<sup>1,2</sup>. Here we describe the first known unequivocal embryonic remains of sauropod dinosaurs—the only known non-avian dinosaur embryos from Gondwana—from a nesting ground in the Upper Cretaceous stage of Patagonia, Argentina. At this new site, Auca Mahuevo (Fig. 1), thousands of eggs are distributed over an area greater than 1 km<sup>2</sup>. The proportion of eggs containing embryonic remains is high: over a dozen *in situ* eggs and nearly 40 egg fragments encasing embryonic material were recovered. In addition to bone, these specimens contain large patches of fossil skin casts, the first definitive portions of integument ever reported for a non-avian dinosaur embryo. As morphology of the eggs enclosing these osseous and integumentary remains is identical, we propose that these specimens belong to the same sauropod species. This discovery allows the confident association of the megaloolithid type of dinosaur eggshell<sup>3</sup> with sauropod dinosaurs.

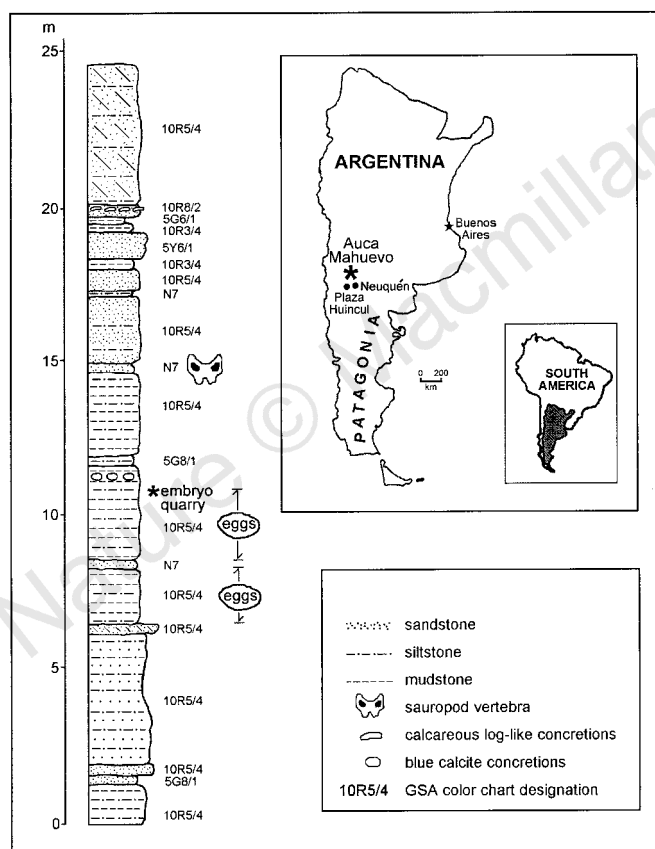
Embryonic bone is typically found flattened on the bottom of the egg. Only the shafts of postcranial elements are preserved in the Auca Mahuevo specimens prepared so far, and few cranial elements are easily identifiable from a mass of diagenetically fused bone. Nevertheless, cranial remains from two specimens (PVPH-112 and PVPH-113) allow a confident taxonomic identification of these embryos. Loose, pencil-like teeth are distinct (Fig. 2). PVPH-112 has at least 32 teeth, with one complete tooth measuring 2.0 mm. The teeth have straight margins that gradually taper towards the crown's apex. Crowns are formed by smooth enamel that lacks denticles or primary ridges. The absence of crown denticles is considered to be a synapomorphy of neosauropods<sup>4</sup> (these include the common ancestor of *Saltasaurus* and *Diplodocus* plus all its descendants<sup>4,5</sup>). Among dinosaurs, pencil-like teeth are known from diplodocid, dicreosaurid and titanosaur neosauropods<sup>4</sup>. The

The left postorbital preserved in PVPH-112 (Fig. 2) has an inverted L-shape with a long, tapering jugal process, a depressed and rostrally expanded anterior process, and a short, blunt squamosal process. It is similar to that of sauropod dinosaurs<sup>8,9</sup>. A thin, elongate jugal process is regarded as diagnostic of sauropods<sup>10</sup>. Portions of the skull roof are visible in both PVPH-112 and PVPH-113. The skull roof of PVPH-113 exhibits a large, expanded bone plate, slightly wider than long, with concave sides (Fig. 2). We interpret this plate to represent diagenetically fused frontals. Its concave sides formed the dorsal rims of the orbits. Frontals that are wider than long are considered a synapomorphy of sauropods<sup>10</sup>. The

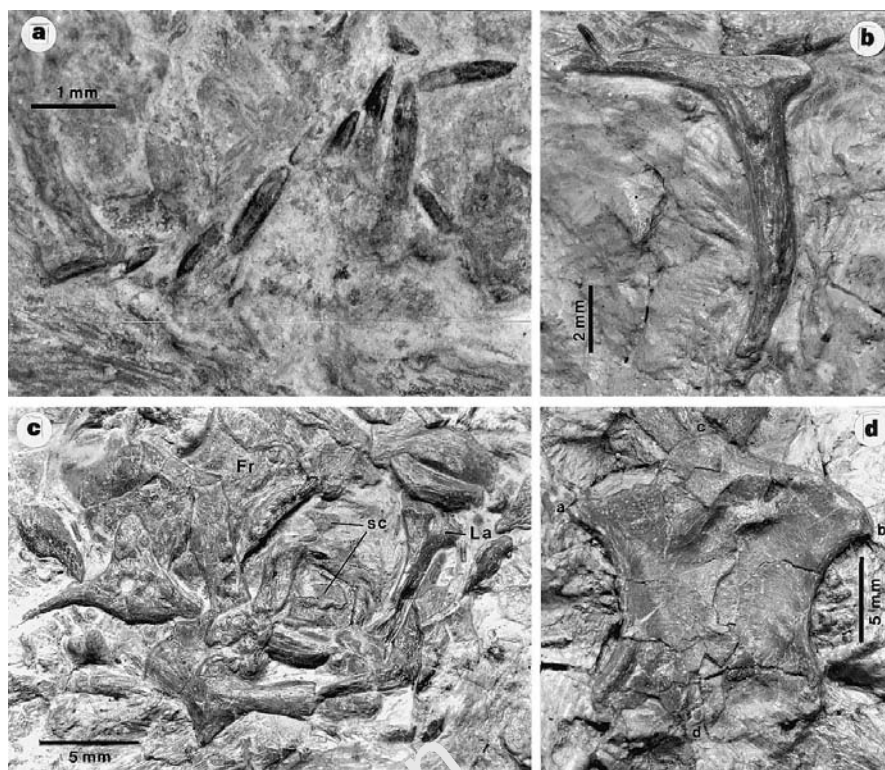
Numerous eggshell fragments collected on the erosion surface at Auca Mahuevo contained skin patches of varying size. Only a few non-avian dinosaurs preserve remnants of their body covering. Integumentary remains have been reported for embryos tentatively assigned to therizinosaurid theropods, but the attribution of these remains to skin is not definitive<sup>11</sup>. Petrographic analysis of the Auca Mahuevo specimens showed the presence of clay, quartz and a carbonate mineral, indicating that the preserved skin is the cast of the original integument. The general skin pattern consists of round, non-overlapping, tubercle-like scales of subequal size ( $\sim 300\text{ }\mu\text{m}$  in diameter) (Fig. 3). PVPH-126 contains a stripe formed by three rows of larger scales. The subrectangular-shaped scales of the central row ( $\sim 800\text{ }\mu\text{m}$ ) are twice as large as those of the lateral rows ( $\sim 400\text{ }\mu\text{m}$ ). Because these skin casts do not overlie articulated bones, it is impossible to establish their precise anatomical location. However, the stripe probably ran along the embryo's back. A rosette pattern of scales is present in PVPH-130 (Fig. 3). The circular, central scale ( $\sim 780\text{ }\mu\text{m}$  in diameter) is surrounded by ten smaller scales that have roughly one-half the central scale's diameter. The tuberculated scale pattern of the Auca Mahuevo embryos, forming non-overlapping scales and rosettes, compares well with the pattern known for adult non-avian dinosaur skin<sup>12</sup>. Although dermal spines have been reported for diplodocoid sauropods<sup>12</sup>, our specimens show no evidence of these structures.

The eggs are round, roughly 13–15 cm in diameter, and appear to have been spherical to subspherical in shape (average volume 800 cm<sup>3</sup>), although all are fractured and somewhat compressed. Shell thickness varies from 1.00 mm to 1.78 mm, with an average thickness of 1.40 mm. On less weathered eggshell, surface ornamentation consists of compact, domed tubercles. In some specimens the tubercles coalesce, forming short ridges. The diameter of individual tubercles varies from 0.35 mm to 0.65 mm, with an average diameter of 0.45 mm (Fig. 4). Pore canals for gas exchange are unevenly distributed between the tubercles. The narrow, straight pores (150–200 µm diameter) maintain a constant diameter throughout the eggshell. Occasionally, the pores 'flare' at the shell surface, producing an elliptical, funnel-like opening.

Shell units consist of calcitic, radial-tabular ultrastructure, with an average midsection width of 0.5 mm (Fig. 4). The typically parallel shell-unit margins interlock and extend to the surface in the upper three-quarters of the shell, but are separate and distinct in the lower quarter. Nearly horizontal growth lines cross adjacent shell units, but dip slightly where shell-unit margins separate in the lower, interior portion of the shell.

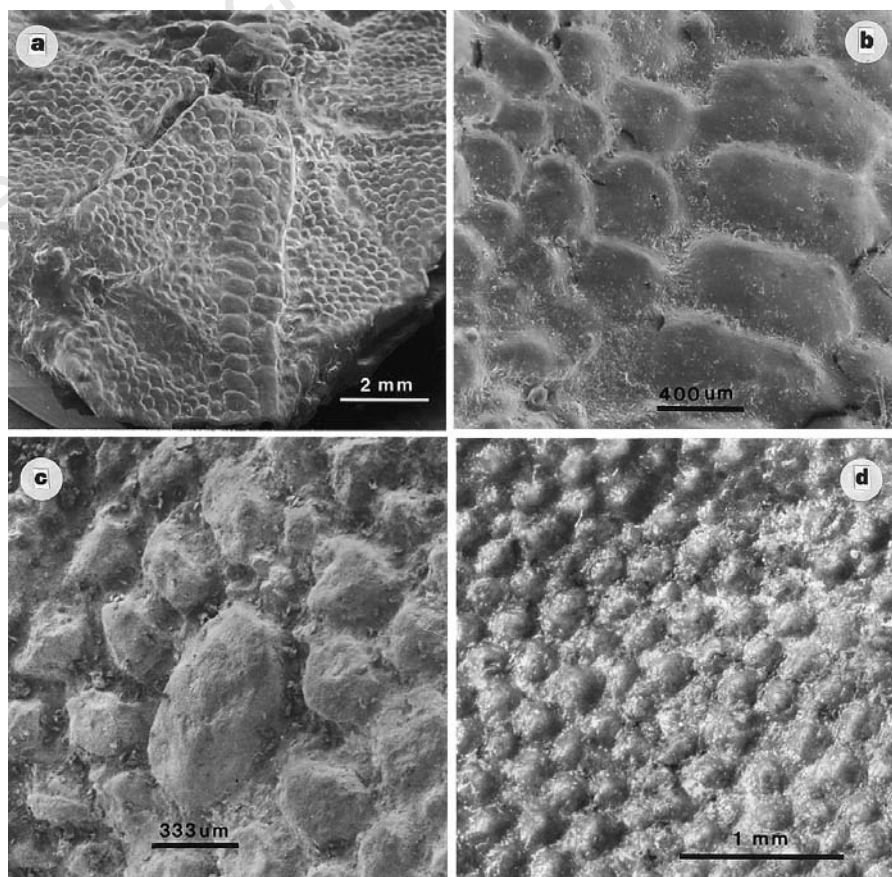


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**Figure 2** Bone morphology of the sauropod embryos from Aucas Mahuevo. **a**, Cluster of teeth of specimen PVPH-112. **b**, Left postorbital in dorsal view (PVPH-112). **c**, Right orbital region in lateral view (PVPH-112). **d**, Frontals of PVPH-

113 in dorsal view; **a** to **b** and **c** to **d** show maximum width and length respectively. Fr, frontal; La, lacrimal; sc, sclerotic plates. PVPH, Paleontología de Vertebrados, Museo Municipal 'Carmen Funes', Plaza Huincul.

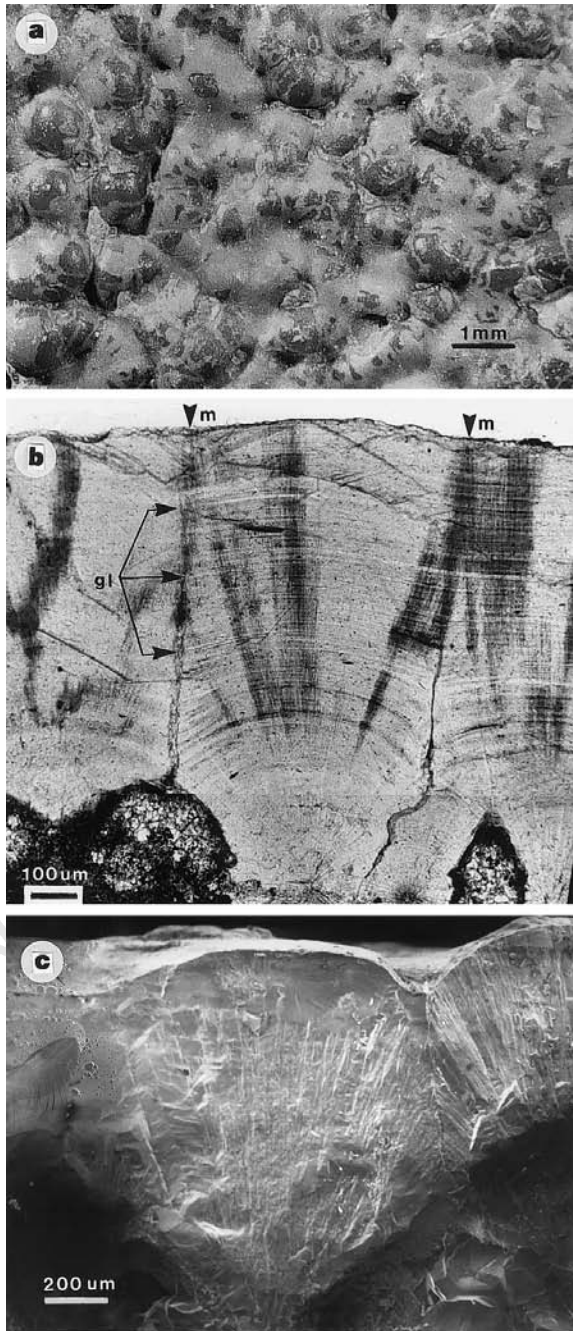


**Figure 3** Natural cast of the integument of the sauropod embryos from Aucas Mahuevo. **a**, Large patch (PVPH-126), showing a triple row of large scales. **b**,

Detail of **a**. **c**, Rosette structure of PVPH-130. **d**, Detail of non-overlapping scales of PVPH-131.



The size, shape, surface ornamentation and eggshell microstructure of these eggs correspond to the Megaloolithidae<sup>3</sup>, a descriptive classification for fossil eggs. Megaloolithid eggs occur in Jurassic deposits of the western United States and Portugal, and the Cretaceous of France, Spain, India, Romania and Argentina<sup>17–20</sup>. Fragmentary eggs of megaloolithid type have been found in another locality of the Río Colorado Formation<sup>21</sup>, roughly 120 km southeast of Auca Mahuevo.



**Figure 4** Eggshell morphology of the Auca Mahuevo embryos. **a**, Surface ornamentation of eggshell showing moderate weathering and tuberculate surface (PVPH-132). Calcite encrusts the surface and fills pore structures. **b**, Radial thin section of megaloolithid eggshell (PVPH-111); shell exterior is to the top, photographed in ordinary light. Note parallel shell units, horizontal growth lines, and weathered exterior. **c**, Stereomicrograph image (SEM) of megaloolithid eggshell unit (PVPH-113). gl, growth lines; m, shell unit margin.

Because of their large size and purported association with sauropod bones, megaloolithid eggs have often been assigned to Sauropoda<sup>20–23</sup>. However, without the definitive evidence of diagnostic embryonic material inside the egg, the assignment of dinosaur eggs to a particular taxon has often proved erroneous<sup>24,25</sup>. The anatomical information of the bones and teeth allows a confident identification of the Auca Mahuevo embryos as sauropod dinosaurs. Previous reports of alleged sauropod embryonic remains were founded on unreliable inferences based primarily on the small size of the specimens<sup>26,27</sup>. (Mohabey's<sup>27</sup> taxonomic identification has been seriously questioned<sup>28</sup>). Thus, our results confirm that certain megaloolithid eggs were laid by sauropods, contradicting previous speculation that sauropod dinosaurs were viviparous<sup>29</sup>.

The limited osteological evidence in the Auca Mahuevo embryos does not allow taxonomic identification beyond Sauropoda. However, their dental morphology places them within Neosauropoda. As titanosaur neosauropods are the only sauropods known from the Río Colorado Formation and the only Late Cretaceous sauropods with pencil-like teeth, these embryos are probably titanosaurs. □

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# Legume-based cropping systems have reduced carbon and nitrogen losses

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In agricultural systems, optimization of carbon and nitrogen cycling through soil organic matter can improve soil fertility and yields while reducing negative environmental impact. A basic tenet that has guided the management of soil organic matter for decades has been that equilibrium levels of carbon and nitrogen are controlled by their net input and that qualitative differences in these inputs are relatively unimportant<sup>1–3</sup>. This contrasts with natural ecosystems in which there are significant effects of species composition and litter quality on carbon and nitrogen cycling<sup>4,5</sup>. Here we report the net balances of carbon and nitrogen from a 15-year study in which three distinct maize/soybean agroecosystems are compared. Quantitative differences in net primary productivity and nitrogen balance across agroecosystems do not account for the observed changes in soil carbon and nitrogen. We suggest that the use of low carbon-to-nitrogen organic residues to maintain soil fertility, combined with greater temporal diversity in cropping sequences, significantly increases the retention of soil carbon and nitrogen, which has important implications for regional and global carbon and nitrogen budgets, sustained production, and environmental quality.

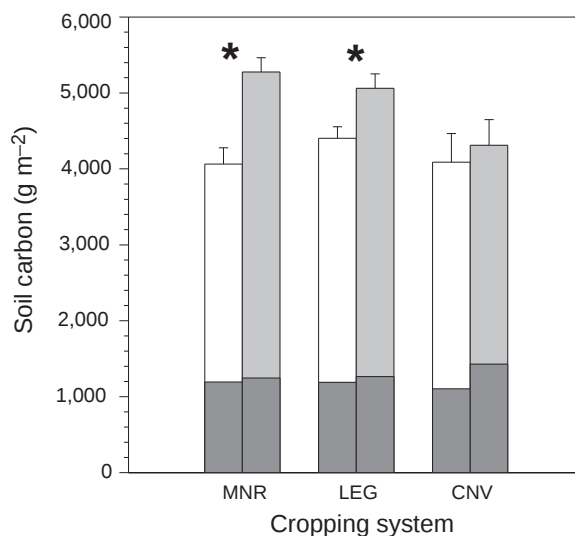
We studied carbon and nitrogen balances in two legume-based and one conventional, fertilizer-driven agroecosystem. The conventional system consisted of a maize/soybean rotation; a mineral nitrogen fertilizer was applied before maize was planted and pesticides were used as needed. The other two cropping systems depended on legumes for nitrogen fixation and were managed on the basis of 'organic' (US) or 'ecological' (Europe) strategies, avoiding the use of synthetic fertilizers and pesticides<sup>6</sup>. One system simulated a beef operation in which crop biomass (legumes and grasses) was fed to beef cattle and the manure was subsequently returned to the field as the primary nitrogen source for maize (MNR system). The other system received nitrogen directly from legumes through incorporation of leguminous biomass before maize planting (LEG system). Maize and soybeans were not present as frequently in these systems as in the conventional system, because small grains and several other legumes were also included in the rotation. Ten-year averages for 1986–95 maize yields were 7,140, 7,100 and 7,170 kg ha<sup>-1</sup> in the MNR, LEG and conventional systems, respectively, and were not significantly different (analysis of variance ANOVA,  $P > 0.5$ ). For the past ten years of the experiment, economic profitability from the three systems has been comparable<sup>7</sup>.

As a result of these distinct management strategies, there were significant quantitative and qualitative differences in organic residue inputs and in soil carbon sequestration. The conventional system had greater mean cumulative above-ground net primary productivity (ANPP, Table 1) and returned more crop residues to the soil than did both of the legume-based systems. The MNR system had the greatest harvest intensity; only 36% ANPP was returned to the soil as crop residues. Total carbon returned to the soil in the MNR and conventional systems, however, was not significantly different, because of steer manure additions in the MNR system (Table 1). The quantity of carbon inputs was not the

major factor affecting soil carbon storage in these cropping systems. Even though the MNR and conventional systems received equal amounts of carbon, only the MNR system showed a significant increase in carbon stored in soil (Table 1). The LEG system, with lower average carbon inputs from above-ground sources, also showed an increase in soil carbon.

In contrast to the conventional system, which received only senescent-crop residues (Table 1), the two legume-based systems received relatively diverse residues that differed in terms of biochemical composition (the residues were from senescent crops, leguminous biomass and/or steer manure). Studies of litter-quality effects using agricultural residues have produced inconsistent results<sup>1–3,8,9</sup>. It is likely that the increased carbon storage in the MNR system is partially due to the return of steer manure to the field. Compared with senescent-crop residues, a larger proportion of manure-derived carbon is retained in soil, probably because manure is already partly decomposed and contains a larger proportion of chemically recalcitrant organic compounds<sup>8,9</sup>. On the other hand these studies did not find significant effects of types of plant species on long-term carbon equilibrium<sup>1,8,9</sup>.

We studied the potential role of plant-species differences on soil carbon storage, using variations in the natural abundance of  $\delta^{13}\text{C}$  associated with photosynthetic pathways to estimate the relative contribution of  $\text{C}_4$  and  $\text{C}_3$  plants to soil organic matter (SOM). Maize, the only  $\text{C}_4$  crop present, accounted for 74%, 48% and 22% of the returned residues in the conventional, LEG and MNR systems, respectively. In the conventional system, maize-derived carbon still replaces the original soil carbon deposited by the  $\text{C}_3$  temperate forests that preceded agriculture in this region. In this case, net soil carbon levels did not change because the loss of  $\text{C}_3$ -derived carbon was nearly equivalent to the gain of  $\text{C}_4$ -derived carbon (Fig. 1). In contrast, the net gains in soil carbon seen in the LEG and MNR systems were due to significant increases in  $\text{C}_3$ -derived carbon. Levels of soil carbon derived from  $\text{C}_4$  plants did not change in the MNR and LEG systems. In the LEG system levels of  $\text{C}_3$ -derived carbon were disproportionately high, accounting for 88% of the net increase in soil carbon although only half of the



**Figure 1** Soil carbon levels in 1981 (left-hand bars) and 1995 (right-hand bars); means  $\pm$  s.e.m. are shown. MNR, manure as N source; LEG, N directly from legumes; CNV, conventional system (see text). The amount of  $\text{C}_4$ -derived carbon is indicated by the dark grey portion of each bar; the remainder of each bar represents  $\text{C}_3$ -derived carbon. Asterisks indicate significant differences in 1995 mean soil carbon levels compared with 1981 levels, ANOVA,  $P < 0.05$ . In addition to residues from maize, the MNR system also receives unknown amounts of  $\text{C}_4$ -derived carbon inputs from the steer manure, making a quantitative comparison of the proportion of  $\text{C}_4$  inputs and  $\text{C}_4$ -derived soil carbon impossible in this system.

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**Table 1 Plant productivity and changes in soil carbon 1981–95**

| Cropping system | Net primary productivity* | Plant residues returned† |        |       | Manure input‡ | Total organic residue input‡ | Change in soil carbon 1981 to 1995§ |
|-----------------|---------------------------|--------------------------|--------|-------|---------------|------------------------------|-------------------------------------|
|                 |                           | Senescent                | Living | Total |               |                              |                                     |
| MNR             | 69a                       | 21                       | 3.7    | 25a   | 19            | 44b                          | 12                                  |
| LEG             | 68a                       | 31                       | 7.5    | 39b   | 0             | 39a                          | 6.6                                 |
| Conventional    | 75b                       | 43                       | 0      | 43c   | 0             | 43b                          | 2.2§                                |

\* Cumulative carbon fixed by annual above-ground net primary productivity; † Carbon in residues returned to the soil; and ‡ net changes (increases) in soil carbon; all over 15 years. Senescent residues are from crops and weeds. Residues incorporated from living plants were mainly of leguminous origin in the LEG system but were predominantly from grasses in the MNR system. Numbers within a column followed by a different letter are significantly different at the 0.05 probability level (protected Scheffe's). § There was no significant change in soil carbon in the conventional system (ANOVA,  $P > 0.05$ ). Values are kg carbon  $\times 10^{-3}$  per ha.

residues returned were from  $C_3$  plants. Replacement of  $C_4$ -derived soil carbon in pools with turnover times of less than 20 years, which accounts for about 5% of the total soil carbon<sup>10</sup>, cannot explain this discrepancy. These changes in the natural abundance of  $\delta^{13}C$  in SOM in the LEG system indicate that differences in plant-species composition have contributed to differential retention of soil carbon. Plant species can affect carbon equilibrium through differences in below-ground net primary productivity (NPP)<sup>11</sup>, the timing and level of root turnover/exudates<sup>12</sup>, litter quality<sup>4</sup>, tendencies to foster the formation of soil aggregates<sup>13</sup>, and changes in microbial community structure and function<sup>14,15</sup>.

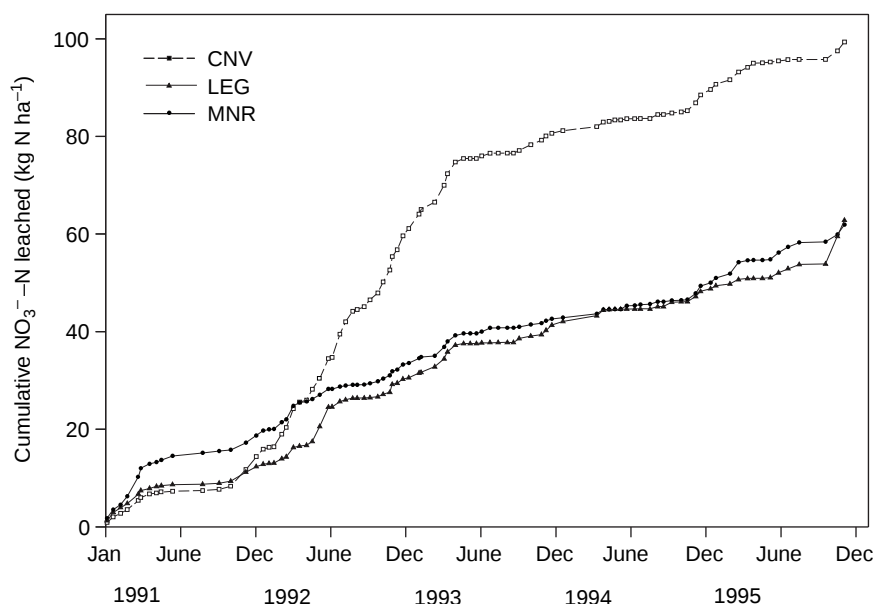
Qualitative differences in nitrogen inputs also had a major influence on nitrogen retention in these agroecosystems. Nitrogen losses due to leaching in 1991–95 were comparable in the LEG and MNR systems, averaging 13 kg nitrogen  $ha^{-1} yr^{-1}$ , but were about 50% higher in the conventional system, averaging 20 kg  $ha^{-1} yr^{-1}$ , (ANOVA,  $P = 0.06$ ; Fig. 2). Seasonal effects in all cropping systems were similar, with the greatest losses occurring during the late-fall to early-spring months when mineralization tends to exceed crop demand. Leaching losses were greatest from late fall of 1991 to spring of 1993 compared with the later half of the rotation cycle, particularly in the conventional system.

Cumulative nitrogen additions were similar in the conventional and MNR systems, as were nitrogen exports from non-leguminous crops (Fig. 3a, b). Over the course of 15 years, nitrogen inputs from soil amendments have exceeded exports by crops by a total of 520 kg  $ha^{-1}$  in the conventional and 540 kg  $ha^{-1}$  in the MNR systems (Fig. 3c). Despite these similarities in net balance, there were significant differences in soil nitrogen storage. Most of the surplus nitrogen received by the MNR system over the 15 years can be

accounted for by the significant increase in soil nitrogen from 1981 to 1995 (Fig. 3d), whereas in the conventional system soil nitrogen levels have decreased since 1981 (Fig. 3d).

Nitrogen inputs into the LEG system are more difficult to quantify because nitrogen fixed by the green manure was the major nitrogen input. However, we have estimated the maximum nitrogen input from the green manure over 15 years to be 840 kg nitrogen  $ha^{-1}$ . If the proportion of nitrogen fixed ranged from 75% to 100%, nitrogen inputs from the green manure would have been 630–840 kg  $ha^{-1}$  (Fig. 3a). Non-leguminous exports were not significantly different from those in the conventional system, but were somewhat lower than those in the MNR system (protected Scheffe's,  $P < 0.05$ ; Fig. 3b). Soil nitrogen levels in this system have not changed significantly (Fig. 3d).

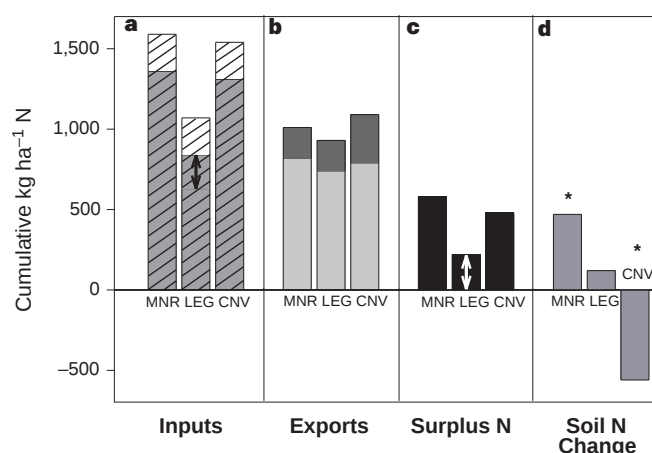
The nitrogen unaccounted for in our balance calculations was +240,  $\pm 100$  and +1,020 kg  $ha^{-1}$  in the MNR, LEG and conventional systems, respectively, and probably reflects differences in gaseous losses and nitrogen fixation by soybeans not included in our calculations. Although we have no measurements of gaseous losses, we can estimate the potential impacts of soybeans on nitrogen balance on the basis of data from the experiment. Nitrogen balances suggest that nitrogen fixation was probably lower in conventional-system soybeans and greater in LEG-system soybeans. Calculations for possible nitrogen-fixation scenarios in the LEG and conventional systems show that even large differences in soybean nitrogen-fixation rates between systems did not alter the basic trends in nitrogen balance. For example, if nitrogen fixed by soybeans were 75% of total soybean nitrogen in the LEG system, the net nitrogen input from senescent roots and shoots would have been only 60–140 kg  $ha^{-1}$  over 15 years. Likewise, nitrogen extrac-



**Figure 2** Cumulative nitrate leaching during 1991 to 1995. Means of data from lysimeters installed in three entry points of four replicates are shown. Cumulative

nitrogen leached over five years was significantly different across cropping systems (ANOVA,  $P = 0.06$ ).





**Figure 3** Comparison of cumulative nitrogen inputs and exports and changes in soil nitrogen storage after 15 years. **a**, Nitrogen inputs under the control of the manager (grey with stripes represents nitrogen input from steer manure, leguminous biomass or mineral fertilizers) and estimated environmental nitrogen inputs (white with stripes represents atmospheric nitrogen deposition and nitrogen fixation by free-living bacteria). **b**, Exports of nitrogen from non-leguminous crops (light grey) combined with nitrate leaching losses (dark grey). **c**, Nitrogen surplus based on inputs minus exports. **d**, The change in soil nitrogen is the difference between nitrogen contents in the plough layer for 1995 compared to 1981; asterisks indicate statistical significance, ANOVA,  $P < 0.05$ . Arrows in LEG-system bars indicate the possible range of nitrogen-fixation inputs.

tion by soybeans in the conventional system can account for only part of the missing 1,020 kg of nitrogen (surplus + change in soil nitrogen); that is, a nitrogen-fixation rate of 40% of soybean total nitrogen would result in the extraction of 280–350 kg ha<sup>-1</sup> over 15 years, a fairly typical outcome for highly determinant soybeans in maize rotations<sup>16</sup>.

These results for nitrogen parallel our findings for carbon, indicating that quantitative differences in nitrogen balance were not the major factor affecting soil nitrogen retention. Instead, qualitative differences in the form of nitrogen inputs and subsequent effects on internal nitrogen cycling had a significant impact on long-term soil nitrogen retention. Detailed microplot studies using <sup>15</sup>N as a tracer showed that there are differences in the partitioning of nitrogen from organic versus mineral sources, with more legume-derived nitrogen than fertilizer-derived nitrogen immobilized in microbial biomass and SOM<sup>17,18</sup>. If immobilization is lower in the conventional system compared with in the other two systems, this may explain the greater leaching of NO<sub>3</sub><sup>-</sup> that we observed in this system. Differences in plant-species composition may contribute to reducing NO<sub>3</sub><sup>-</sup> leaching by scavenging soil nitrogen during periods in which summer cash crops, such as maize and soybeans, are not active<sup>19</sup>.

Our results show that even in these intensively managed agroecosystems, plant-species composition and litter quality influence SOM turnover markedly. Increases in SOM in the MNR and LEG systems were highly significant in terms of ecosystem function and soil quality. Greater retention of both carbon and nitrogen suggests that use of low carbon-to-nitrogen residues to maintain soil fertility combined with increased temporal diversity restores the biological linkage between carbon and nitrogen cycling in these systems and could lead to improved global carbon and nitrogen balances. Application of these practices in the major maize/soybean growing region in the USA would increase soil carbon sequestration by 0.13–0.30 × 10<sup>14</sup> g yr<sup>-1</sup>. This is equal to 1–2% of the estimated annual carbon released into the atmosphere from fossil fuel combustion in the USA<sup>20</sup> (1.4 × 10<sup>15</sup> g carbon yr<sup>-1</sup>, 1994) and is a significant contribution considering that the USA has agreed to

reduce average CO<sub>2</sub> emissions to 7% below 1990 levels by 2008–2012 as part of the Kyoto Protocol. In addition, CO<sub>2</sub> emissions from the two legume-based systems are lower than emissions from the conventional system because of a 50% reduction in energy use<sup>21</sup>. The potential effects on the nitrogen cycle are much greater in magnitude because the fixed nitrogen used in agricultural activities is responsible for a 60% increase in global levels of biologically active nitrogen<sup>22</sup>. Reduced nitrogen losses combined with increased soil nitrogen storage will lead to reductions in the amount of nitrogen that must be applied to maintain yields.

## Methods

The experiment covered 6 ha and consisted of a randomized, complete block design. Details of experimental design and farming practices are described elsewhere<sup>23</sup>. Carbon contents of organic residues are calculated from plant biomass data collected from 1981–1995 assuming a carbon content of 42% on a dry weight basis.

**Soil analyses.** Composite soil samples collected in 1981 and 1995 were analysed for total carbon and nitrogen with the Leco CN-2000 analyser (Leco Corporation). Natural abundance of <sup>13</sup>C was determined by combustion of triplicate samples with a Europa Scientific carbon–nitrogen analyser connected to a Europa Scientific Tracermass mass spectrometer. To estimate the original <sup>13</sup>C natural abundance before the introduction of C<sub>4</sub> plants, we collected a composite soil sample along two transects from a forested, never-farmed site located ~0.5 km from the experiment. The proportion of carbon derived from corn residues, C<sub>4</sub>%, was calculated for 1981 and 1995 as  $C_4\% = (\delta_m - \delta_f) / (\delta_{mr} - \delta_f) \times 100$ , where  $\delta_m$  =  $\delta^{13}C$  content of soil after maize cultivation;  $\delta_f$  = -25.58‰ the  $\delta^{13}C$  content of soil under the nearby native mixed hardwood forest; and  $\delta_{mr}$  = -13.1‰ the  $\delta^{13}C$  content of maize residues<sup>24</sup>.

**Nitrogen budget.** Nitrogen inputs from leguminous green manures were calculated by using above-ground biomass nitrogen content data and adjusting for below-ground contributions using root biomass data and nitrogen content from the experiment in 1997. Values for total nitrogen fixation in these plants were taken from published studies to estimate the minimum proportion of nitrogen fixed<sup>16,25</sup>. Soybeans, which were present in all three cropping systems, were not included in nitrogen-budget calculations because of the potential for variability in nitrogen fixation by soybeans<sup>16</sup>. However, we used our data on soybean yields, residue returns and nitrogen contents to estimate potential impacts on nitrogen balance. Usually, no more than 50% of total soybean nitrogen is derived from nitrogen fixation and generally two-thirds of the total nitrogen is exported from the beans<sup>3,16</sup>. Nitrogen fixation by free-living bacteria, which represents a minor contribution to nitrogen inputs in agricultural systems using tillage, was assumed to be 5 kg nitrogen ha<sup>-1</sup> yr<sup>-1</sup> (ref. 26). In earlier work we did not find significant differences in the potential for nitrogen fixation by free-living organisms in these cropping systems<sup>27</sup>. We estimated nitrogen inputs from atmospheric deposition to be 15 kg nitrogen ha<sup>-1</sup> yr<sup>-1</sup> on the basis of data for northeastern USA<sup>28</sup>. Estimates of nitrate leaching for the 15-year period were based on five years of data collected in this experiment during 1991–95 from intact-core below-ground lysimeters installed in 4 replicates × 3 entry points, for a total of 36 lysimeters (each 0.45 m<sup>2</sup> in area)<sup>29</sup>.

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## Motion integration in a thalamic visual nucleus

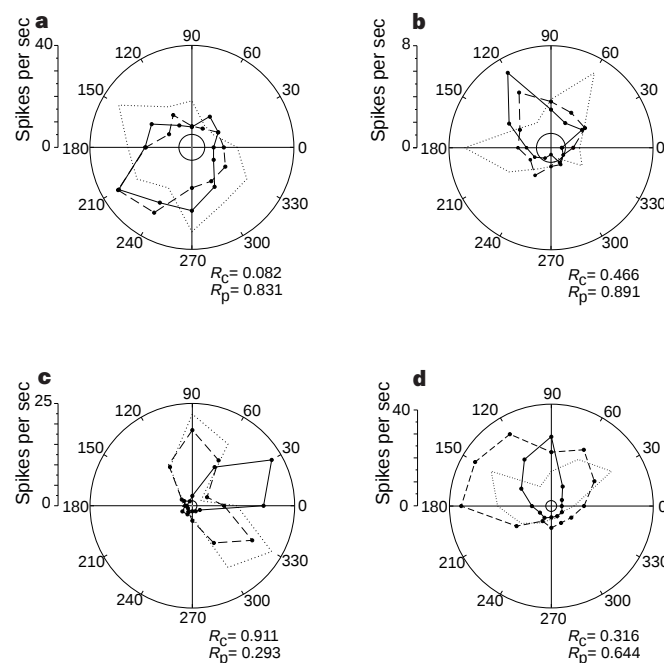
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Thalamic nuclei have long been regarded as passive relay stations for sensory information en route to higher level processing in the cerebral cortex. Recently, physiological and theoretical studies have reassessed the role of the thalamus and it has been proposed that thalamic nuclei may actively participate with cortical areas in processing specific information<sup>1–4</sup>. In support of this idea, we now show that a subset of neurons in an extrageniculate visual nucleus, the lateral-posterior pulvinar complex, can signal the true direction of motion of a plaid pattern, indicating that thalamic cells can integrate different motion signals into a coherent moving percept<sup>5–8</sup>. This is the first time that these computations have been found to occur outside the higher-order cortical areas<sup>5,6,9,10</sup>. Our findings implicate extrageniculate cortico-thalamo-cortical loops in the dynamic processing of image motion, and, more generally, as basic computational modules involved in analysing specific features of complex visual scenes.

The integration of motion signals is usually considered to be a two-stage process<sup>5,11</sup>. The first stage involves the analysis of object features as one-dimensional components, which are integrated at a second stage. It has been argued that the first stage is inherently limited in its coding of local motion signals (the aperture problem<sup>5</sup>), and that the second stage, combining the outputs from the first, is necessary to generate a global percept of an object in motion. This

second stage has been attributed to cortical networks, as have all forms of higher-order processing. However, models have been proposed in which thalamic nuclei participate in these processes, interacting closely with the neocortex<sup>1–4,12–14</sup>. A common implication of these models is that cells on both sides of the cortico-thalamic loop have similar higher-order response properties, although there has been no clear demonstration of subcortical neurons responding to higher-order visual stimuli. In well-developed visual systems, the pulvinar region is a likely candidate for a subcortical counterpart to the cortex where a loop involved in the analysis of moving objects could be established. The pulvinar complex represents a higher-order nucleus because it receives its major input from layer V cortical neurons, rather than directly from retinal ganglion cells; it is also in reciprocal communication with virtually all visual and associative cortical areas<sup>15,16</sup>. This region is often associated with visual attention<sup>17,18</sup> and visually guided movement<sup>19</sup>, but theories of its function remain speculative<sup>15</sup>. In cats, the physiological response properties in the lateral-posterior pulvinar (LP-pulvinar) complex indicate that these cells code attributes of image motion such as direction, velocity, and the relative motion between an object and its background<sup>20</sup>. On the basis of these response properties and connectivity patterns, we considered that the LP-pulvinar complex could participate in analysing the global motion of complex scenes. We therefore studied the sensitivity of cells in the cat's LP-pulvinar complex to moving plaid patterns. This stimulus comprises two superimposed drifting gratings differing only in orientation. A human observer perceives a single rigid pattern moving unambiguously with a direction and velocity uniquely consistent with the constraints imposed by the motion of the individual components<sup>5,7,8</sup>. At the neuronal level, a cell that is selective for the global motion of the plaid pattern responds with a profile similar to that of a single grating moving in the integrated direction ('pattern'-motion selectivity), rather than



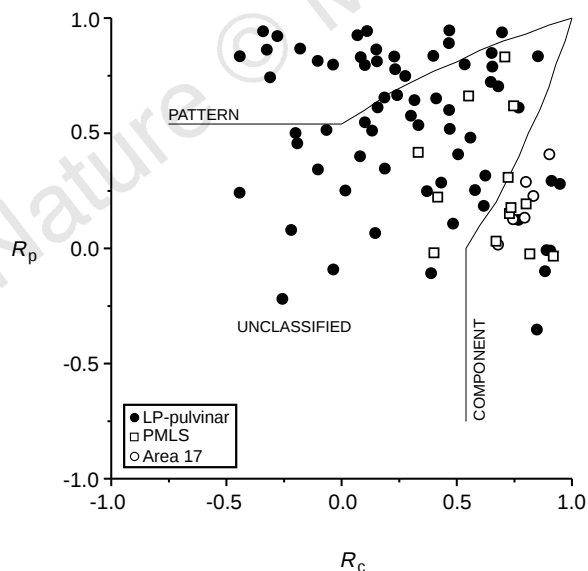
**Figure 1** Polar graphs illustrating the responses of LP-pulvinar neurons to gratings (solid line) and plaid patterns (dashed line) drifting in 12 directions of motion. We considered the response to gratings alone as the predicted profile for a truly pattern-motion selective unit. The dotted line represents the predicted response to plaids for a component-motion selective unit. **a, b**, Pattern-motion selective neurons. **c**, The response of a component-motion selective cell. **d**, The discharges of an unclassified direction-selective cell. The small central circles represent spontaneous activity levels.

in the directions of the oriented components comprising the pattern ('component'-motion selectivity)<sup>5,6</sup>.

We recorded from 67 direction-selective units in the LP-pulvinar complex, and found a subset of 21 cells that responded unequivocally to the pattern motion of plaids. For the two cells illustrated in Fig. 1a, b, there is a close correspondence between the directional tuning function computed from the responses to moving gratings and plaids, indicating that these neurons are able to signal the pattern's true direction of motion. Another subset of seven LP cells (10.5%) gave bilobed tuning curves, with peaks roughly symmetrical to the peak obtained in the single grating experiment (Fig. 1c). These responses closely match the tuning curve predicted for component responses, indicating that these neurons process the one-dimensional motion signals making up the plaid pattern. The remaining 39 units (58.2%) could not be grouped in either of the two categories (unclassified direction-selective cells; Fig. 1d).

We classified the cells' responses to plaids on the basis of the calculation of partial correlation coefficients, comparing the responses to plaids to the predictions of component and pattern motion<sup>8</sup> (Fig. 2). These data show that a substantial proportion of neurons (~31%) in the LP-pulvinar complex lie in the region found to be selective to pattern motion. Component responses were also present, indicating that both local and global information is processed at the thalamic level. As a comparison, we measured the responses to moving plaid patterns from cells in the primary visual cortex (area 17) and in the posteromedial part of the lateral suprasylvian cortex (a region often referred to as the functional analogue of the primate middle temporal (MT) area<sup>21</sup>). We found that most neurons in these areas were component-motion selective and that there was no pattern-motion direction selective cells, consistent with previous reports<sup>6,22</sup>.

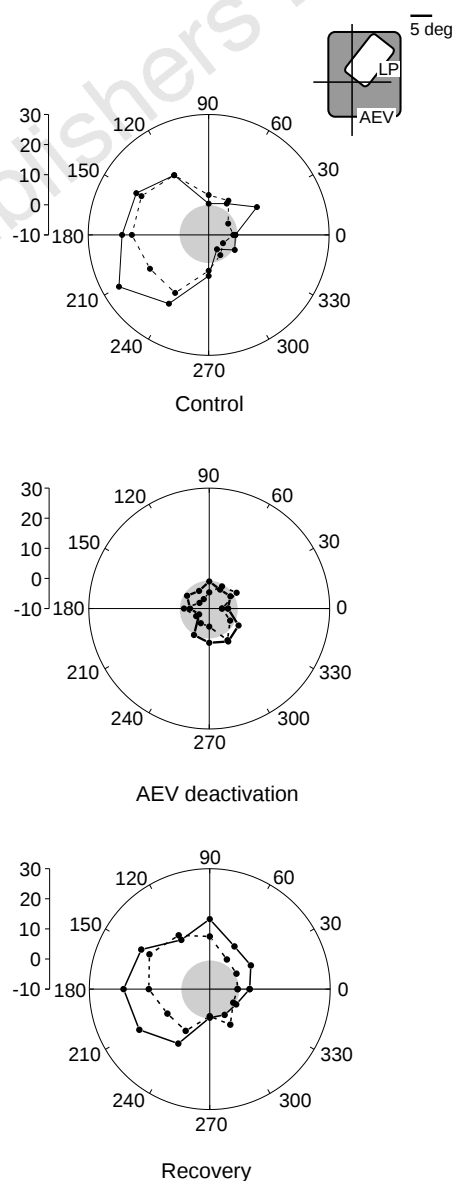
Histological reconstruction of the electrode tracks indicated that most pattern-motion selective cells in the LP-pulvinar complex (80%) were located in the medial part of the lateral posterior nucleus (LPm) whereas the remaining units were found in the



**Figure 2** Scatter plot of partial correlations for pattern and component selectivity: filled symbols, neurons in the LP-pulvinar; open circles, area 17; and open squares, PMLS cortex. Each direction-selective unit was classified by quantifying the degree of correspondence between the response to plaid and the responses predicted from the pattern (responses to the grating alone) and the component models (that is, shifting the grating responses by 60° in both directions and summing the resulting curves around the clock). The data space is divided into three statistical regions. Cells falling in the upper left and lower right areas are respectively pattern- and component-motion selective. The points lying in between represent unclassified direction-selective cells.

lateral section of this nucleus. The medial area corresponds to the tecto-recipient zone of the LP-pulvinar, which receives a major input from the superior colliculus and establishes reciprocal connections with the anterior ectosylvian visual (AEV) cortex<sup>15,23</sup>. This extrastriate area is the only region of the cat visual cortex described as possessing a population (~55%) of pattern-motion-processing cells<sup>6</sup> similar to those observed in primate area MT<sup>8-10</sup>. The presence of pattern-motion selective cells on both sides of the AEV-LPm loop raises the possibility that this cortico-thalamic network is a module specifically involved in the processing of motion information.

We performed two sets of experiments investigating the physiological link between these two regions. First, we measured the responses of 16 LP neurons to plaids before and after pharmacological deactivation of visuotopically corresponding regions of the AEV cortex. Eight LP cells were classified as pattern selective, of



**Figure 3** Effect of deactivating the AEV cortex on a pattern-motion selective neuron in the medial part of the lateral posterior nucleus. During cortical deactivation, a strong suppression of the cell's overall response and a concomitant loss of its ability to signal direction of the drifting grating (solid line) and plaids pattern (dashed line) was observed (blocking index of -0.25 and -0.4, respectively). In each polar graph, data points within the shaded region represent cell discharges below spontaneous activity levels. The inset shows the location of the receptive field of the thalamic neuron in reference to the AEV scotoma.



which half were affected by cortical deactivation: for three of these cells, the plaid responses were strongly reduced (Fig. 3); for the fourth they were enhanced (data not shown). These results demonstrate that the lateral posterior nucleus and AEV cortex are functionally linked and, furthermore, that this loop is likely to be involved in processing higher-order motion information. However, the finding that the responses of four pattern-motion selective units were not altered by the cortical deactivation suggests that pattern selectivity in LP-pulvinar does not depend solely on descending projections from the AEV (see below). Blocking the AEV cortex also reduced the responses to plaids for three out of five component cells and two out of three unclassified direction-selective units. That component cells were affected by cortical deactivation is surprising, given that almost no such cells were found in the AEV<sup>6</sup>. One explanation is that local velocity signals from the lateral posterior nucleus are used by the AEV for integrating or comparing local and global motion signals. Alternatively, deactivation of the AEV might affect lower-level motion areas, which in turn project to the LP nucleus. This finding may also indicate that simultaneous excitation of both regions is needed for information to be processed along the cortico-thalamic loop<sup>24</sup>. In the present case, disrupting the feed-

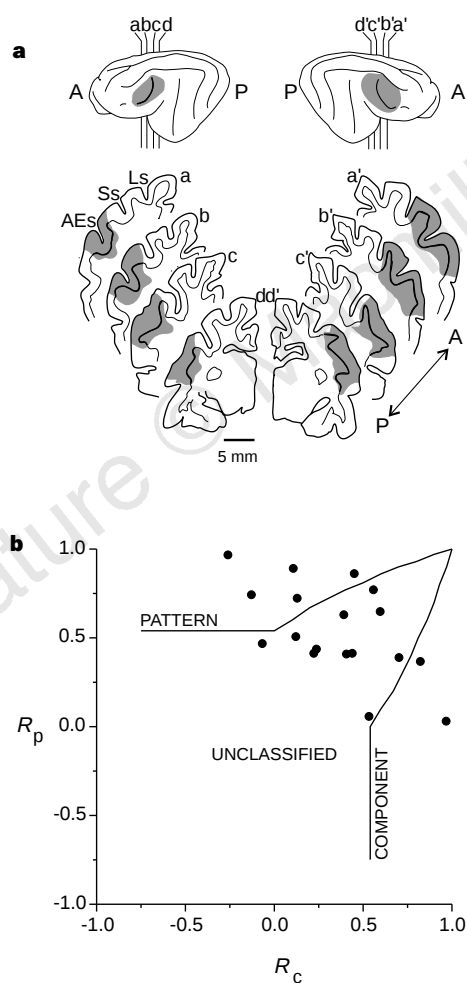
back input short-circuits the loop and decreases the probability of thalamic firing.

To determine whether pattern motion responses in LP-pulvinar neurons depend solely on inputs arising from the AEV, we recorded in the extrageniculate thalamus of three cats with acute bilateral ablation of the anterior ectosylvian cortex (see Methods). In all animals, we could still record pattern-motion selective responses (Fig. 4). These data, together with the fact that cortical deactivation does not affect half of the pattern-motion cells, indicate that LP-pulvinar neurons can process pattern motion from inputs other than those provided by the AEV cortex. To test this, we studied pattern-motion sensitivity in the LP-pulvinar complex of cats with ablation of the AEV and the lateral suprasylvian cortex, generally considered to be the main motion area in cats<sup>21</sup>, although no pattern-selective cells have been found in the lateral suprasylvian subdivisions investigated<sup>22</sup>. Ablation of the two cortices yields a global reduction in the visual responsiveness of LP cells, and in the number of direction-selective neurons. Testing with drifting gratings showed only three of 16 units to prefer a specific direction of motion; responses to plaids of all cells were unclassified direction-selective. Thus, the persistence of pattern-motion selectivity in LP neurons after removal of the AEV cortex appears to depend on the integrity of the lateral suprasylvian cortex. These data support the proposition<sup>25</sup> that there is a subpopulation of lateral suprasylvian neurons that can code the true direction of moving objects. It is also possible that intrinsic computations are taking place within the thalamus (for example, integration of multiple-component signals). Moreover, the absence of pattern-motion cells indicates that subcortical inputs from the superior colliculus do not account for pattern selectivity in the LP-pulvinar complex. This observation agrees with previous reports showing that deactivation of the superior colliculus does not alter the responses in the medial part of the lateral posterior nucleus<sup>15</sup>, and that this region does not contain pattern-motion units<sup>8</sup>.

The finding that LP neurons can code the integrated motion of plaid patterns indicates that the LP-pulvinar complex is important in motion integration, and also supports the theoretical notion that clusters of thalamic cells, in conjunction with cortical assemblies, participate in the analysis of complex percepts<sup>1,3,4</sup>. One possibility is that sensory maps in the AEV and lateral suprasylvian are used to establish a 'global' template in the LP-pulvinar complex. Through feedback loops, these maps could be used dynamically to compare the first and second stages of motion processing of an image encoded at cortical levels with the information stored at the thalamic level<sup>1,10</sup>. In addition, successive iterations within the cortico-thalamic loops could help to refine the cortical image (for example, segmentation from motion).

Our findings may force a reconsideration of the computational steps involved in the analysis of moving scenes, which so far have been modelled only at the cortical level<sup>5,9,26</sup>. Whereas the current 'two-stage processing' models have only been described in the primate visual system, there is evidence that they may also be present in the cat. Indeed, it has been shown that higher-order motion computations do occur in the cat extrastriate cortex<sup>6</sup>. Furthermore, computer-based analyses of connectivity patterns have indicated that intermediate cortical stages of motion analysis<sup>26</sup> are likely to be present in areas of the lateral suprasylvian cortex that have not yet been fully explored (for example, the posterolateral and anterolateral parts)<sup>6,25</sup>. It remains to be determined whether the same pattern of responses described here also exists in the primate thalamic visual areas. The reciprocal connections between the pulvinar and middle temporal area<sup>27</sup> and the strong similarities in the organization of cat and primate extrageniculate pathways<sup>28</sup> support this view. This suggests that these cortico-thalamic loops represent a common module of computational organization in these species.

In conclusion, our demonstration that higher-order properties



**Figure 4** The effects of cortical ablations. **a**, Coronal sections showing the site and extent of the cortical ablations of one cat at various rostrocaudal H-C coordinates. Top, lateral view of the two hemispheres showing the location of the lesions a-d, a'-d'. **b**, Distribution of partial correlation coefficients of LP-pulvinar neurons of cats with bilateral ablation of the AEV cortex. Note the presence of all cell types and in particular, the pattern-motion selective units. Abbreviations: A, anterior; AEs, anterior ectosylvian sulcus; Ls, lateral sulcus; P, posterior; Ss, suprasylvian sulcus.

are analysed outside the neocortex is important for the reassessment of the role of the thalamus and cortico–thalamic loops<sup>1,3,4,8,9,13</sup>. That neuronal responses to pattern motion can be found in the LP-pulvinar complex in anaesthetized preparations indicates that the function of thalamic nuclei goes beyond the mere relaying of sensory information in relation to the state of vigilance. We propose that the LP-pulvinar complex acts as a platform for processing and integrating specific aspects of an image in cooperation with the visual cortex. □

## Methods

Cats were initially anaesthetized with acepromazine (1.0 mg per kg body weight) and atropine (0.2 mg per kg); general anaesthesia was carried out using a gaseous mixture of halothane (1–3%) and N<sub>2</sub>O/O<sub>2</sub> (50/50%). The animal was paralysed by intravenous injection of gallamine triethiodide (10 mg per kg body weight per hour) and artificially ventilated (N<sub>2</sub>O/O<sub>2</sub>:70/30% and halothane 0.5%). Core temperature, electrocardiogram and electroencephalogram were continuously monitored. Pupils were dilated with atropine and nictitating membranes were retracted with phenylephrine hydrochloride (2.5%). The eyes were protected using contact lenses of appropriate refractive power. Animals were treated in accordance with the guidelines of the Canadian Council for the Protection of Animals. Varnished tungsten microelectrodes were used to record single-unit activity in LP-pulvinar cells. Neuronal activity was fed to a computer for peristimulus time histogram acquisition. Stimulation was carried out using drifting sinusoidal gratings and plaid patterns (Picasso image synthesizer; frame rate of 200 Hz) presented on a CRT (Data Check 5117; mean luminance of 14 cd m<sup>-2</sup>, z axis gamma correction) placed 57 cm in front of the animal and subtending 28 × 28° of visual angle. Plaids were generated by a frame-interleaved method and composed of two superimposed sine-wave gratings differing in orientation (120°) and of identical spatial frequency, temporal frequency and contrast. Both plaids and gratings were presented for at least 4 complete trials consisting of 12 interleaved directions of motion in 30° increments. Responses to plaids were classified as pattern-selective or component-selective by calculating partial correlations using the following equation<sup>8</sup>:  $R_p = (r_p - r_c r_{pc}) / [(1 - r_c^2)(1 - r_{pc}^2)]^{1/2}$ .  $R_p$  represents the partial correlation coefficient for the pattern prediction,  $r_c$  is the correlation coefficient of the plaid response calculated from the component model,  $r_p$  is the correlation coefficient for the plaid response from the pattern model and  $r_{pc}$  is the correlation coefficient for the two models. Similarly,  $R_c$  is the partial correlation defined for the component-selective prediction and is calculated by exchanging  $r_p$  with  $r_c$  in the equation. A cell is considered as pattern-motion selective when the value of  $R_p$  is significantly greater than either  $R_c$  or zero<sup>8</sup>. Electrolytic lesions were made along recording tracks and cell localization was determined.

**Deactivation experiments.** In eight experiments, a glass microelectrode filled with  $\gamma$ -aminobutyric acid (GABA, 200  $\mu$ M, stained with 1% Chicago sky blue) was lowered into the AEV. It was inserted in the headstage of a nanopump (WPI) modified to allow simultaneous recording. Placement of the electrode in AEV was determined on the basis of stereotaxic coordinates and visual cues. The deactivation procedure was performed only if there was a visuotopic correspondence between the receptive fields in lateral posterior nucleus and AEV cortex. All LP receptive fields were located between +12 and -15° for elevation and -5 and +20° for azimuth. Cortical activity was continuously recorded before, during, and after the injection. The deactivation solution was injected and continuously delivered at a rate ranging between 80 and 90 nl min<sup>-1</sup> for the first 2 min and maintained at 20–30 nl min<sup>-1</sup> throughout the testing period. The mean ( $\pm$ s.d.) volume injected was 460 ( $\pm$ 140) nl, and the mean diameter of these injections inferred from Chicago sky blue staining<sup>29</sup> was 1.2 ( $\pm$ 0.2) mm. Tested LP cells were kept for further analysis only if the injection of GABA yielded a clear suppression of the AEV multi-unit activity and if the location and extent of the injection site could be determined. For each cell, a blocking index was calculated by dividing the mean response of the LP unit during deactivation by the mean control response recorded before blocking. Indices of zero and one would indicate, respectively, that cell discharges were either totally abolished or not affected. Details of the deactivation technique are given elsewhere<sup>29</sup>.

**Cortical ablations.** In three experiments, we recorded from the LP-pulvinar complex in cats with acute bilateral lesioning of the AEV, that is, the anterior ectosylvian sulcus including the entire visual area. The dura was resected and

the cortex was removed by aspiration of the grey matter lying along the ventral bank of the anterior ectosylvian sulcus and overlying the claustrum<sup>23</sup>. In two additional experiments, the lateral suprasylvian cortices were also removed in conjunction with the AEV. The cortex located in the medial and lateral banks of the suprasylvian sulcus (the six subregions defining the lateral suprasylvian cortex<sup>30</sup>) was ablated by aspiration. The cavities were filled with sterile gelfoam and sealed with warm agar and wax. Recordings in the LP-pulvinar complex were made after a 12–16 hour period of recuperation following the surgical procedures. To determine whether the trauma associated with the destruction of both the AEV and the lateral suprasylvian cortices had caused a general depression of brain activity, we first recorded from a few neurons in the lateral geniculate nucleus, superior colliculus and primary visual cortex. In all of these regions, cell properties were similar to those observed in intact animals.

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# K<sup>+</sup> is an endothelium-derived hyperpolarizing factor in rat arteries

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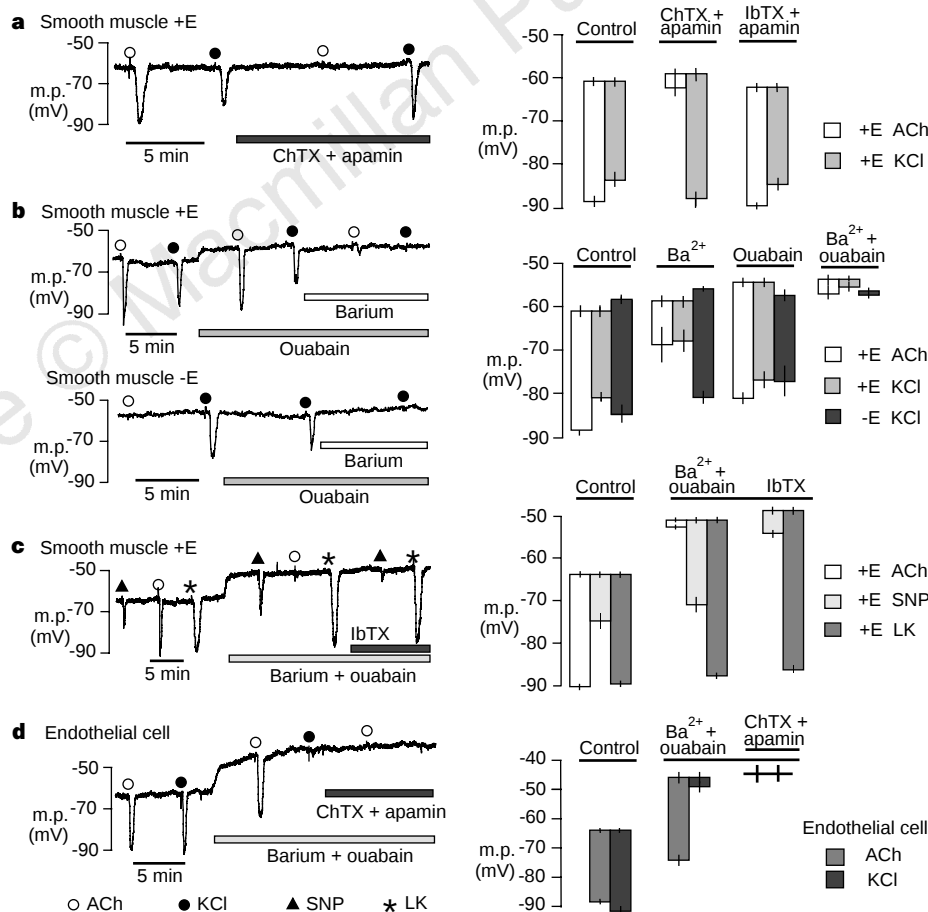
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In arteries, muscarinic agonists such as acetylcholine release an unidentified, endothelium-derived hyperpolarizing factor (EDHF) which is neither prostacyclin nor nitric oxide<sup>1–3</sup>. Here we show that EDHF-induced hyperpolarization of smooth muscle and relaxation of small resistance arteries are inhibited by ouabain plus Ba<sup>2+</sup>; ouabain is a blocker of Na<sup>+</sup>/K<sup>+</sup> ATPase<sup>4</sup> and Ba<sup>2+</sup> blocks inwardly rectifying K<sup>+</sup> channels<sup>5</sup>. Small increases in the amount of extracellular K<sup>+</sup> mimic these effects of EDHF in a ouabain- and Ba<sup>2+</sup>-sensitive, but endothelium-independent,

manner. Acetylcholine hyperpolarizes endothelial cells and increases the K<sup>+</sup> concentration in the myoendothelial space; these effects are abolished by charybdotoxin plus apamin. Hyperpolarization of smooth muscle by EDHF is also abolished by this toxin combination, but these toxins do not affect the hyperpolarization of smooth muscle by added K<sup>+</sup>. These data show that EDHF is K<sup>+</sup> that effluxes through charybdotoxin- and apamin-sensitive K<sup>+</sup> channels on endothelial cells. The resulting increase in myoendothelial K<sup>+</sup> concentration hyperpolarizes and relaxes adjacent smooth-muscle cells by activating Ba<sup>2+</sup>-sensitive K<sup>+</sup> channels and Na<sup>+</sup>/K<sup>+</sup> ATPase. These results show that fluctuations in K<sup>+</sup> levels originating within the blood vessel itself are important in regulating mammalian blood pressure and flow.

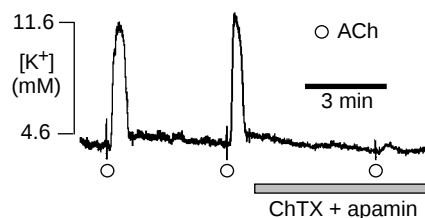
Vasorelaxation induced by EDHF is abolished by charybdotoxin plus apamin but not by iberiotoxin plus apamin<sup>6,7</sup>. In vascular endothelial cells, K<sup>+</sup> channels that are sensitive to charybdotoxin and apamin are present<sup>8</sup> and their activation by bradykinin releases EDHF<sup>9</sup> and stimulates K<sup>+</sup> efflux<sup>10</sup>. As increases in the extracellular K<sup>+</sup> concentration, [K<sup>+</sup>]<sub>o</sub>, in the range 6–16 mM evoke rapid arteriolar relaxation and hyperpolarization<sup>5,11</sup>, we proposed that the stimulation of endothelial cells by agonists<sup>12</sup> might activate apamin- and charybdotoxin-sensitive K<sup>+</sup> channels. Any resulting K<sup>+</sup> efflux might then hyperpolarize the adjacent smooth muscle, revealing EDHF to be endothelium-derived K<sup>+</sup>.



**Figure 1** Profiles of EDHF- and K<sup>+</sup>-induced hyperpolarizations in hepatic artery. **a**, In endothelium-intact (+E) arteries, smooth-muscle hyperpolarizations induced by EDHF (released by 10  $\mu$ M acetylcholine, ACh) but not by K<sup>+</sup> were abolished by charybdotoxin (ChTX) + apamin (100 nM each). Iberiotoxin (IbTX) + apamin (100 nM each) had no effect. **b**, In endothelium-intact arteries, EDHF- and K<sup>+</sup>-induced smooth-muscle hyperpolarizations were reduced by ouabain (1 mM) and abolished by then adding Ba<sup>2+</sup> (30  $\mu$ M). Initial exposure to Ba<sup>2+</sup> (30  $\mu$ M) alone reduced EDHF- and K<sup>+</sup>-induced smooth-muscle hyperpolarizations, which were abolished when ouabain (1 mM) was also added. In endothelium-denuded

arteries (–E), EDHF was not released and K<sup>+</sup>-induced hyperpolarizations were abolished only by Ba<sup>2+</sup> + ouabain. **c**, Responses of endothelium-intact arteries to sodium nitroprusside (SNP; 1  $\mu$ M) were insensitive to Ba<sup>2+</sup> + ouabain but inhibited by IbTX (100 nM). Levocromakalim (LK, 10  $\mu$ M)-induced hyperpolarizations were unaffected by these inhibitors. **d**, Endothelial cells became hyperpolarized in response to ACh and K<sup>+</sup> but Ba<sup>2+</sup> + ouabain blocked responses to K<sup>+</sup> only. Further addition of ChTX + apamin abolished responses to ACh. Left, responses in single vessels; right,  $n = 5–12$ ; means  $\pm$  s.e.m. are shown. m.p., membrane potential.





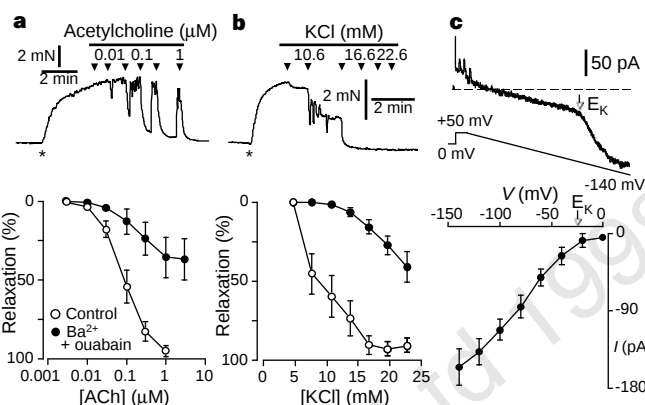
**Figure 2** Determination of the myoendothelial  $K^+$  concentration in endothelium-intact hepatic arteries using a  $K^+$ -sensitive microelectrode. Acetylcholine ( $10 \mu M$ ) induced a reproducible, rapid increase in  $K^+$  concentration that was inhibited by  $100 \text{ nM}$  charybdotoxin (ChTX) +  $100 \text{ nM}$  apamin. The basal  $K^+$  concentration in the myoendothelial space was always slightly lower than that of the bathing solution (bathing solution  $K^+$  concentration  $4.6 \text{ mM}$ ).

To test this hypothesis, we compared the effects of EDHF (released by acetylcholine) and of increasing  $[K^+]_o$  on membrane potential in rat hepatic artery, a blood vessel that shows typical responses to EDHF<sup>7</sup>. In 27 smooth-muscle cells, the membrane potential ( $-60.9 \pm 0.3 \text{ mV}$ ) was hyperpolarized both by EDHF (membrane potential  $-87.7 \pm 0.5 \text{ mV}$ ) and by a  $5\text{-mM}$  increase in  $[K^+]_o$  to  $9.6 \text{ mM}$  (membrane potential  $-81.1 \pm 0.7 \text{ mV}$ ).  $K^+$ -induced hyperpolarizations were unaffected by charybdotoxin plus apamin (when  $5 \text{ mM}$   $K^+$  was added, the change in membrane potential was  $23.0 \pm 1.9 \text{ mV}$  before and  $28.9 \pm 2.9 \text{ mV}$  after addition of charybdotoxin plus apamin;  $n = 5$ ) whereas those induced by EDHF were abolished by charybdotoxin plus apamin ( $28.0 \pm 1.4 \text{ mV}$  before,  $3.4 \pm 1.6 \text{ mV}$  after;  $n = 5$ ) but were unaffected by iberiotoxin<sup>13</sup> plus apamin (Fig. 1a). These results indicate that acetylcholine may induce  $K^+$  efflux by opening endothelial cell  $K^+$  channels that are sensitive to charybdotoxin and apamin, and that the large-conductance,  $Ca^{2+}$ -sensitive  $K^+$  channels<sup>13</sup> are not involved.

We then determined how endothelial  $K^+$  efflux hyperpolarized the muscle. Studies of vascular myocytes<sup>5,11,14</sup> showed that an increase in  $[K^+]_o$  stimulated a  $Ba^{2+}$ -sensitive, inwardly rectifying  $K^+$  current ( $I_{K(IR)}$ ) or a ouabain-sensitive  $Na^+/K^+$  ATPase. When we inhibited  $Na^+/K^+$  ATPase with ouabain<sup>4</sup>, EDHF- and  $K^+$ -induced hyperpolarizations were only slightly reduced (Fig. 1b). However, additional blockade of inwardly rectifying  $K^+$  channels ( $K_{IR}$ ) with  $30 \mu M$   $Ba^{2+}$  (a specific inhibitory concentration)<sup>11,15</sup> abolished hyperpolarizations in response to both stimuli (Fig. 1b). When we reversed the order of application of  $Ba^{2+}$  and ouabain,  $Ba^{2+}$  alone reduced EDHF- and  $K^+$ -induced hyperpolarizations, which were abolished when ouabain was also present (Fig. 1b). The  $Ba^{2+}$  plus ouabain combination was specific, as it did not reduce hyperpolarizations in response to either sodium nitroprusside (a nitric oxide donor<sup>16</sup>) or levromakalim (which opens the ATP-sensitive  $K^+$  channel<sup>17</sup>) (Fig. 1c). These results again support the hypothesis that EDHF is  $K^+$  and indicate that a  $5\text{-mM}$  increase in  $[K^+]_o$  can activate smooth-muscle  $Na^+/K^+$  ATPase and  $K_{IR}$ .

When we removed the endothelium from the arteries, acetylcholine had no effect but  $K^+$ -induced hyperpolarizations were not diminished (Fig. 1b). However, they were reduced by either  $Ba^{2+}$  or ouabain and abolished in the presence of both substances (Fig. 1b). These results further indicate that EDHF and  $K^+$  share a common identity (Fig. 1b).

We confirmed that charybdotoxin and apamin inhibit EDHF release (Fig. 1a) by measuring the membrane potential of endothelial cells. Acetylcholine markedly hyperpolarized endothelial cells (Fig. 1d), an effect that was insensitive to  $Ba^{2+}$  plus ouabain (control hyperpolarization,  $24.9 \pm 0.3 \text{ mV}$ ;  $Ba^{2+}$  plus ouabain,  $28.0 \pm 2.1 \text{ mV}$ ;  $n = 5$ ) but eliminated when charybdotoxin plus apamin were also present (Fig. 1d). Conversely, acetylcholine-induced, endothelium-dependent hyperpolarization of smooth muscle was always abolished by  $Ba^{2+}$  plus ouabain (Fig. 1b).



**Figure 3** Comparison of EDHF- and  $K^+$ -induced relaxations and identification of inwardly rectifying  $K^+$  currents ( $I_{K(IR)}$ ) in hepatic artery. **a**, **b**, EDHF (**a**, released by the application of increasing concentrations of acetylcholine) and  $K^+$  (**b**) relaxed vessels that had been precontracted with phenylephrine (asterisk,  $0.3 \mu M$ ); these effects were antagonized in the presence of  $30 \mu M$   $Ba^{2+}$  +  $1 \text{ mM}$  ouabain ( $n = 9-13$ ). **c**, Top, whole-cell currents were generated using a reverse-ramp protocol with a quasipathological ( $4.6 \text{ mM}/120 \text{ mM}$ )  $K^+$  gradient and show inward rectification at potentials more negative than  $E_K$ , the  $K^+$  equilibrium potential. Bottom, the component of the inwardly rectifying current that is sensitive to  $30 \mu M$   $Ba^{2+}$  was measured in a separate series of experiments ( $60 \text{ mM}$  extracellular  $K^+$ ;  $n = 5$ ). Means  $\pm$  s.e.m. are shown.

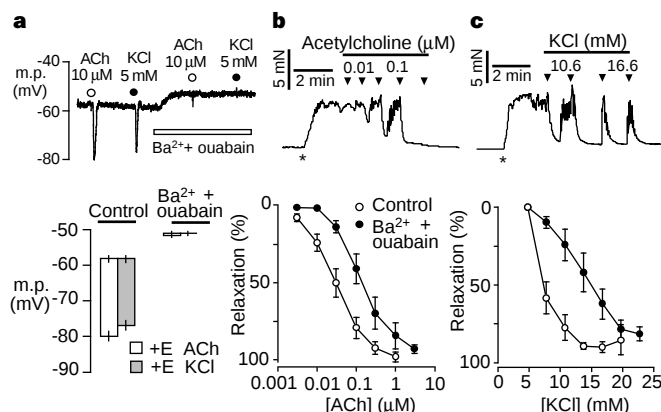
Acetylcholine thus opens charybdotoxin- and apamin-sensitive  $K^+$  channels on the endothelium and EDHF is identical to the  $K^+$  that effluxes through these channels.

To determine whether acetylcholine could stimulate sufficient endothelial  $K^+$  efflux to activate the adjacent muscle, we used  $K^+$ -sensitive microelectrodes. In four hepatic arteries with an intact endothelium, exposure to  $10 \mu M$  acetylcholine raised the  $K^+$  concentration in the myoendothelial space by  $5.9 \pm 1.0 \text{ mM}$  (range  $3.0-7.6 \text{ mM}$ ), an increase that was almost completely abolished ( $0.4 \pm 0.1 \text{ mM}$ ) by apamin plus charybdotoxin (Fig. 2). These results confirm agonist-induced stimulation increases myoendothelial  $K^+$  concentrations sufficiently to activate smooth muscle  $K_{IR}$  and  $Na^+/K^+$  ATPase and indicate that  $K^+$  fluctuations originating within the blood vessel itself are important in the physiological regulation of vascular diameter.

We further investigated the similarity between EDHF and  $K^+$  by measuring tension changes<sup>18</sup> in phenylephrine-contracted segments of hepatic artery. These segments relaxed in a concentration-dependent manner in response to EDHF; this effect was mimicked by increasing  $[K^+]_o$  (Fig. 3a, b). In both cases,  $Ba^{2+}$  plus ouabain inhibited relaxations (Fig. 3b) but not control responses to  $0.3 \mu M$  levromakalim ( $100.6 \pm 6.6\%$  relaxation,  $n = 3$ ).

The fact that  $Ba^{2+}$  (as well as ouabain) was required to inhibit relaxation and hyperpolarization of smooth-muscle cells in response to EDHF and  $K^+$  indicated that  $K_{IR}$  channels must be present on hepatic-artery cells. Not all small arteries express functional  $K_{IR}$  channels<sup>14</sup> and this may explain why ouabain alone can inhibit EDHF in some vessels<sup>4</sup>. However, we could detect  $I_{K(IR)}$  in hepatic-artery cells (Fig. 3c). This current was activated at potentials close to the calculated  $K^+$  equilibrium potential and was rapidly and reversibly abolished when  $30 \mu M$   $Ba^{2+}$  was added to the bath solution.

Many mammalian blood vessels that show  $K^+$ -induced hyperpolarization and relaxation also exhibit responses to EDHF<sup>11,14,19</sup>. We extended our studies to determine whether an increase in  $[K^+]_o$  could explain the effects of EDHF in rat mesenteric artery<sup>18</sup>. Using sharp microelectrodes, we found that the smooth-muscle basal membrane potential ( $-57.7 \pm 0.9 \text{ mV}$ ) became hyperpolarized in response to either acetylcholine (membrane potential  $-79.8 \pm 1.3 \text{ mV}$ ) or increased  $[K^+]_o$  (increase in  $[K^+]_o$



**Figure 4** Comparison of the effects of EDHF and  $K^+$  in small mesenteric arteries with an intact endothelium (+E). **a**, The hyperpolarizing effects of EDHF (released by application of acetylcholine, ACh) and  $K^+$  were abolished by exposure to 1 mM ouabain + 30  $\mu$ M  $Ba^{2+}$  ( $n = 5$ ). **b**, **c**, Relaxations induced in response to EDHF and  $K^+$  in arteries that had been precontracted with phenylephrine (asterisk, 3  $\mu$ M) were inhibited by  $Ba^{2+}$  + ouabain (upper panels, single vessels; lower panels,  $n = 10$ ).

of 5 mM to 9.6 mM; membrane potential  $-76.8 \pm 1.3$  mV).  $Ba^{2+}$  plus ouabain abolished these effects (Fig. 4a). Raising  $[K^+]_o$  from 4.6 mM to 22.8 mM relaxed phenylephrine-contracted segments of mesenteric artery with intact endothelium in a manner similar to that of EDHF. The relaxations were antagonized by  $Ba^{2+}$  plus ouabain but not abolished (Fig. 4b, c) which may reflect the presence of myoendothelial gap junctions<sup>20</sup>. If the importance of these gap junctions increases when the tissue is contracted<sup>21</sup>, hyperpolarization of endothelial cells by acetylcholine or added  $K^+$  (Fig. 1d) could be conducted to the smooth muscle in a  $Ba^{2+}$ - and ouabain-insensitive manner. In support of this idea, the maximum  $K^+$ -induced relaxation of endothelium-denuded segments of mesenteric artery in the presence of  $Ba^{2+}$  plus ouabain was markedly less than when the endothelium was intact (no endothelium, control  $80.0 \pm 11.9\%$ ,  $Ba^{2+}$  plus ouabain  $30.6 \pm 13.7\%$ ,  $n = 5$ ; with endothelium, control  $90.6 \pm 5.5\%$ ,  $Ba^{2+}$  plus ouabain,  $83.8 \pm 11.5\%$ ,  $n = 9$ ).

It has been proposed that EDHF is an epoxyeicosatrienoic acid<sup>22</sup> and that an unknown hyperpolarizing substance is present in luminal perfusates of coronary arteries<sup>23</sup>. However, the agents/extracts involved activate large-conductance,  $Ca^{2+}$ -activated  $K^+$  channels. These channels are not involved in responses to EDHF, as iberiotoxin (which inhibits these channels) cannot substitute for charybdotoxin in inhibition of hyperpolarization (Fig. 1a) and relaxation<sup>7,24</sup>. The release of nitric oxide induced by acetylcholine cannot explain our results, as a nitric oxide synthase inhibitor was always present in our studies. Furthermore, and consistent with the results of a study using nitric oxide<sup>25</sup>, sodium nitroprusside, which releases nitric oxide, produced some hyperpolarization of hepatic artery, an effect largely abolished by iberiotoxin (Fig. 1c).

Our identification of EDHF as  $K^+$  is likely to be universally valid because the characteristics of EDHF in the arteries used are identical to those observed in many other vessels, including those from humans<sup>2,3</sup>. The accumulation of  $K^+$  around resistance vessels following  $K^+$  efflux from muscle and neurons modulates blood flow in the skeletal muscle and coronary and cerebral circulations<sup>26–28</sup>. Our results show that this physiological regulation of vessel tone also extends to  $K^+$  ions that originate from within the walls of the blood vessels themselves.

## Methods

**Microelectrode and tension measurements.** Mesenteric and hepatic arteries from male Sprague–Dawley rats (100–250 g body weight) were used.

Vessels were maintained at 37°C in a physiological salt solution (PSS)<sup>29</sup> containing indomethacin (10  $\mu$ M) and  $N^G$ -nitro-L-arginine (hepatic artery, 300  $\mu$ M; mesenteric artery, 100  $\mu$ M) and were gassed with 95%  $O_2$ :5%  $CO_2$ . Endothelial cells were removed from the vessels if necessary by exposing the lumen to distilled water for 20 s. For microelectrode measurements we used vessels mounted in a bath (volume 3 ml) superfused with PSS (3 ml min<sup>-1</sup>).  $Ba^{2+}$ , ouabain and toxins were added to the PSS supplying the bath; acetylcholine,  $K^+$ , sodium nitroprusside and levromakalim were applied to the flowing PSS to give transient exposure to the stated concentrations. Muscle membrane potential was recorded by impaling cells through the adventitia. Removal of an arterial side branch created a hole through which endothelial cells on the opposite wall could be impaled.  $K^+$  activity in the myoendothelial space was determined using  $K^+$ -sensitive microelectrodes<sup>30</sup> without a shielding pipette. Electrodes were first calibrated and then inserted into an endothelial cell, resulting in a large voltage change. The electrode was then advanced slowly until a sudden voltage reduction indicated its emergence into the myoendothelial space. Data were recorded using conventional amplifiers (WPI Instruments) and digitized with a MacLab system (AD Instruments). Tension studies were performed on 2-mm-long arterial segments (diameter 100–200  $\mu$ m) mounted in a Mulvany–Halpern myograph as described<sup>7,18</sup>. Agents were added to the 10-ml bath under static conditions.

**Whole-cell patch-clamp recordings.** Myocytes from hepatic arteries were dispersed using collagenase and pronase<sup>29</sup>.  $Ca^{2+}$ -free bath solution contained (in mM): NaCl, 124.7; KCl, 4.8;  $MgCl_2$ , 1.2;  $KH_2PO_4$ , 1.2; glucose, 11; HEPES, 10; pH 7.30; gassed with  $O_2$ . Pipette solution contained (in mM): NaCl, 5; KCl, 120;  $MgCl_2$ , 1.2;  $K_2HPO_4$ , 1.2; HEPES, 10; glucose, 11; oxaloacetic acid, 5; sodium pyruvate, 2; sodium succinate, 5; pH 7.30. For cell stimulation and for data recording and analysis, we used pCLAMP 5.5 and Axograph 3.5.5 software (Axon Instruments)<sup>28</sup>.

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## Fringe is essential for mirror symmetry and morphogenesis in the *Drosophila* eye

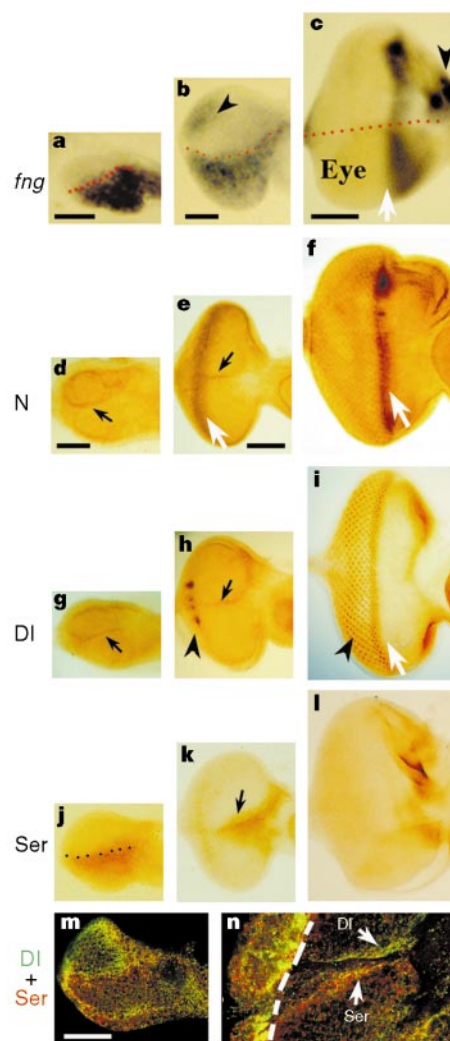
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An early event in *Drosophila* eye development is the division of the eye disc into dorsoventral domains. The dorsoventral pattern is displayed in the adult compound eye as a distinct mirror symmetry across the dorsoventral midline or equator<sup>1,2</sup>. The dorsoventral axis is also implicated in organizing early development of the eye, as retinal differentiation is initiated at the posterior dorsoventral midline<sup>3</sup>. Here we show that Fringe is expressed specifically in the ventral half of the undifferentiated eye disc, thus creating a dorsoventral boundary. Ectopic Fringe borders that are generated by clones of *fringe*<sup>-</sup> cells can reverse the planar polarity of photoreceptor clusters, indicating that the Fringe boundary is crucial for the induction of mirror symmetry. Lack of a Fringe boundary disrupts equatorial expression of Notch signalling proteins and causes a complete failure of eye development. Our results indicate that the formation of the Fringe boundary and subsequent Notch signalling at the equator are essential for organizing mirror symmetry and eye morphogenesis.

Fringe (Fng), a signalling protein with sequence homology to the glycosyltransferases<sup>4</sup>, is restricted to the dorsal compartment in the wing disc, and the border between the *fng*<sup>+</sup> and *fng*<sup>-</sup> cells is crucial for wing outgrowth<sup>5</sup>. Fng regulates the interaction between Notch and its ligands, Serrate (Ser) and Delta (Dl), thereby activating Notch at the dorsoventral (DV) boundary<sup>6–10</sup>. Vertebrate Fng homologues are similarly important in mediating signalling between dorsal and ventral cells in the limb<sup>9</sup>.

In the eye, *fng* messenger RNA was restricted to the ventral domain in the first-instar discs (Fig. 1a), consistent with inversion of the DV axis in the eye–antenna disc compared to the leg and wing discs<sup>11</sup>. In the late second-instar disc, low *fng* expression was seen in the dorsal as well as the ventral domain (Fig. 1b). By late third instar, *fng* was detected in both DV regions anterior to the wave of differentiation marked by the morphogenetic furrow<sup>2</sup>. The early ventral specificity of *fng* led us to examine the expression of Notch and its ligands in early eye discs. Notch was preferentially expressed in the equatorial region before the initiation of differentiation (Fig. 1d). As the eye disc differentiated, Notch was detected in both DV regions posterior to the furrow, while gradually losing its equatorial expression (Fig. 1e, f). Dl and Ser were expressed in the

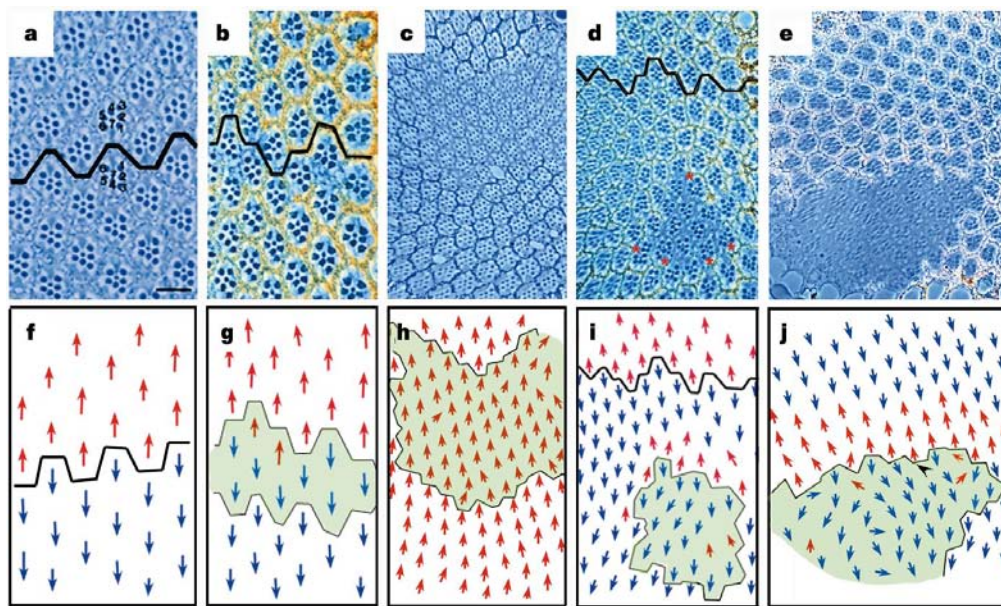


**Figure 1** Expression of *fng* and Notch (N) signalling proteins in eye discs. **a–c**, *fng* mRNA expression. **d–f**, Immunostaining with anti-N (**d–f**), anti-Dl (**g–i**) and anti-Ser (**j–l**) antibodies. Dotted lines indicate DV midline (**a–c**, **j**). Arrowheads indicate weak *fng* expression in dorsal domain (**b**) and expression in the ocelli region (**c**), respectively. Equatorial region and morphogenetic furrow are marked with black and white arrows, respectively (**c–i**, **k**). Expression in differentiating photoreceptors is indicated with black arrowheads (**h**, **i**). **a**, First-instar disc; **b**, late second-instar disc; **d**, **g**, **j**, **m**, early second-instar discs; **e**, **h**, **k**, **n**, early third-instar disc; **c**, **f**, **i**, **l**, late third-instar discs. **m**, **n**, Double labelling of Dl (green) and Ser (red) in second- and early third-instar discs, respectively. Scale bars: **a**, 5  $\mu$ m; **b**, 15  $\mu$ m; **c**, **f**, **i**, **l**, 45  $\mu$ m; **d**, **g**, **j**, 10  $\mu$ m; **e**, **h**, **k**, 20  $\mu$ m; **m**, **n**, 10  $\mu$ m. Dorsal is up and anterior is to the right.

dorsal and ventral regions of the second-instar disc, respectively (Fig. 1g, j, m). The dorsoventral pattern of Dl and Ser was maintained transiently in the equatorial region in the early (Fig. 1h, k), but not in the late (Fig. 1i, l), third-instar disc. Confocal analysis of early third-instar discs identified an equatorial groove in which Ser was preferentially expressed at the ventral ridge, whereas Dl was present in both dorsal and ventral ridges of the groove (Fig. 1n).

To examine the role of *fng* in DV patterning in the eye, we generated *fng*<sup>-</sup> clones by mitotic recombination using  $\gamma$ -irradiation or the yeast FLP recombinase<sup>12</sup>. Two null alleles used, *fng*<sup>13</sup> and *fng*<sup>80</sup>, gave essentially the same results. Each unit eye or ommatidium contains eight photoreceptors clustered in an asymmetric trapezoidal pattern. In the normal eye, ommatidial polarity in the dorsal and ventral halves is opposite (Fig. 2a, f). *fng*<sup>-</sup> clones induced in the





**Figure 2** Polarity reversals by ventral *fng*<sup>-</sup> clones. Sections of wild-type eyes (**a**) and *fng* mosaic eyes (**b-e**). *fng*<sup>-</sup> clones are coloured green (**g-j**). **a, f**, Wild type. Dorsal and ventral trapezoids are indicated by red and blue arrows, respectively. The equator is marked by a black line. R1 to R7 cells are labelled with numbers. **b, g**, *fng*<sup>80</sup> clone at the equator with no polarity reversals. **c, h**, *fng*<sup>80</sup> clone in the dorsal region with no polarity reversals. **d, i**, *fng*<sup>80</sup> clone ~4 rows ventral to the

equator (black line) with polarity reversals (red arrows). Some ommatidia within the clone show altered numbers of photoreceptors (red asterisks). **e, j**, *fng*<sup>80</sup> clone ~8 rows ventral to the equator (located at the top margin of the panel) with polarity reversals in 3-4 rows of ommatidia. An ommatidium with abnormal rotation but normal chirality is marked with a black arrowhead in **j**. Anterior is to the right. Scale bars: **a, b**, 25  $\mu$ m; **c-e**, 50  $\mu$ m.

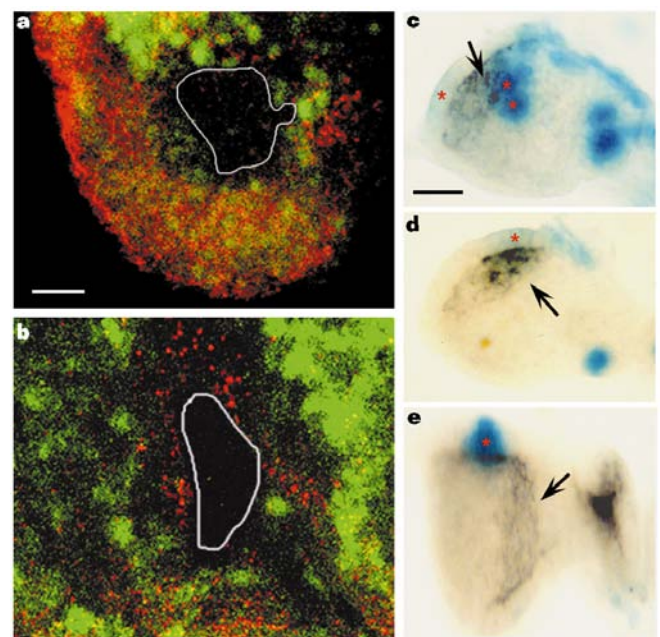
equator or dorsal region did not show significant polarity changes either inside or outside the clone boundary (Fig. 2b, c, g, h). This is consistent with the lack of *fng* expression in the dorsal domain (Fig. 1a). In contrast, up to four rows of ommatidia adjacent to a clone showed DV reversals when clones were induced in the ventral domain, either close to (Fig. 2d, i) or far from (Fig. 2e, j) the equator. This indicates that ectopic *fng* boundaries have a long-range effect on polarity. The extent of polarity reversals was independent of the position of clones in the ventral domain. The shift of chirality was observed only outside the clone boundaries towards the normal equator. The mechanisms underlying this directional polarity reversal require further study. Although most ommatidia within the clones showed normal DV polarity (94% of 101 ommatidia scored from six ventral clones), ~24% of them contained either more or less photoreceptor cells, indicating that *fng* has an additional function during differentiation.

We examined the expression of Notch signalling proteins in second-instar discs containing *fng*<sup>-</sup> clones. At this stage, Fng and Ser are expressed preferentially in the ventral half (Fig. 1). Ser was absent in ventral *fng*<sup>-</sup> clones (Fig. 3a, b), which is consistent with Fng being upstream of Ser in the wing<sup>6</sup>. Some *fng*<sup>-</sup> clones showed enhanced Ser expression on the *fng*<sup>+</sup> side of clone boundary (Fig. 3b), indicating an induction of Ser at an ectopic Fng boundary.

Fng can induce Dl in the *fng*<sup>-</sup> cells by means of Notch activation in the wing<sup>9</sup>. To test this possibility, we generated clones of ectopic Fng in the dorsal region by the FLP-out method<sup>13</sup>. Dl expression was enhanced over long distances from the *fng*<sup>+</sup> clones. *fng*<sup>+</sup> clones within the eye primordium induced Dl expression that was restricted to the posterior to the clone (Fig. 3c), whereas clones at the dorsal polar margin induced Dl up to the DV midline (Fig. 3d), as if the long-range Dl induction was restricted by the equatorial boundary. Occasionally, Dl expression induced by an *fng*<sup>+</sup> clone at the dorsal margin reached the ventral region (Fig. 3e), which can occur if the *fng*<sup>+</sup> clone is formed before the normal equatorial boundary is established. This indicates that the Fng boundary can generate a long-range diffusible signal.

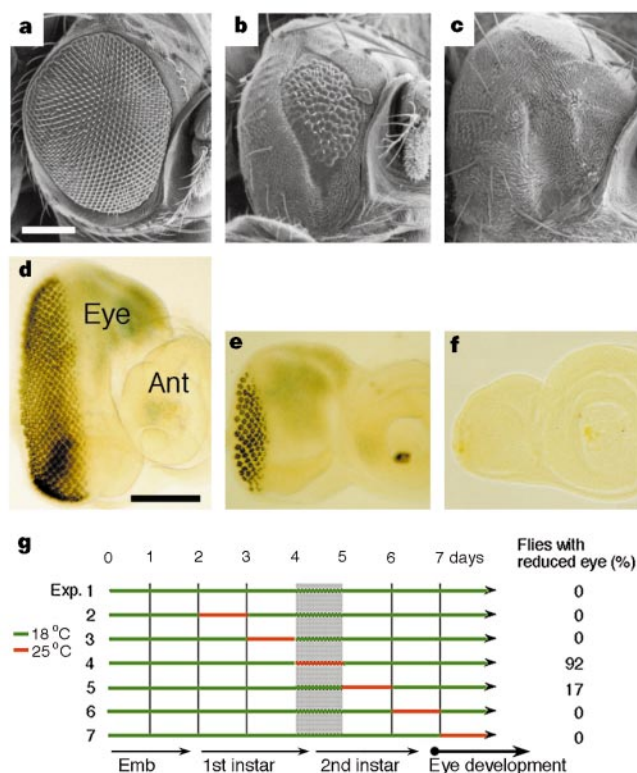
To eliminate the Fng boundary, we induced ubiquitous Fng

expression using the *Gal4/UAS* system<sup>14</sup>. Gal4 expression of the *69B-Gal4* line was detected in both DV regions throughout larval development (data not shown). Transgene expression in the *Gal4/UAS* system depends on temperature<sup>14</sup>. The eyes of *69B-Gal4/UAS-*

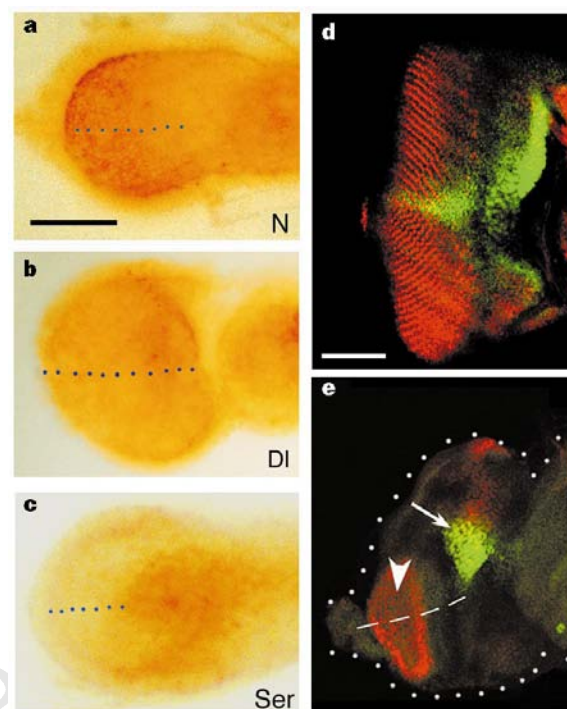


**Figure 3** Ser and Dl expression in *fng* clones. **a, b**, Second-instar eye discs were stained with anti-Myc (green) and anti-Ser (red) antibodies. Clones are identified by the lack of Myc staining and their boundaries are indicated by a solid line. **a**, A ventral *fng*<sup>-</sup> clone with no Ser expression. **b**, A ventral clone with ectopic Ser expression in *fng*<sup>+</sup> cells at the clone boundary. *fng*<sup>-</sup> cells within the clone do not show Ser expression. **c-e**, Increased Dl expression by dorsal *fng*<sup>+</sup> clones. Dl induction (dark brown, arrows) and *fng*<sup>+</sup> clones (blue, asterisks) were detected by anti-Dl and X-gal staining, respectively. Dorsal is up and anterior is to the right. Scale bars: **a**, 10  $\mu$ m; **b**, 4  $\mu$ m; **d-f**, 5  $\mu$ m.

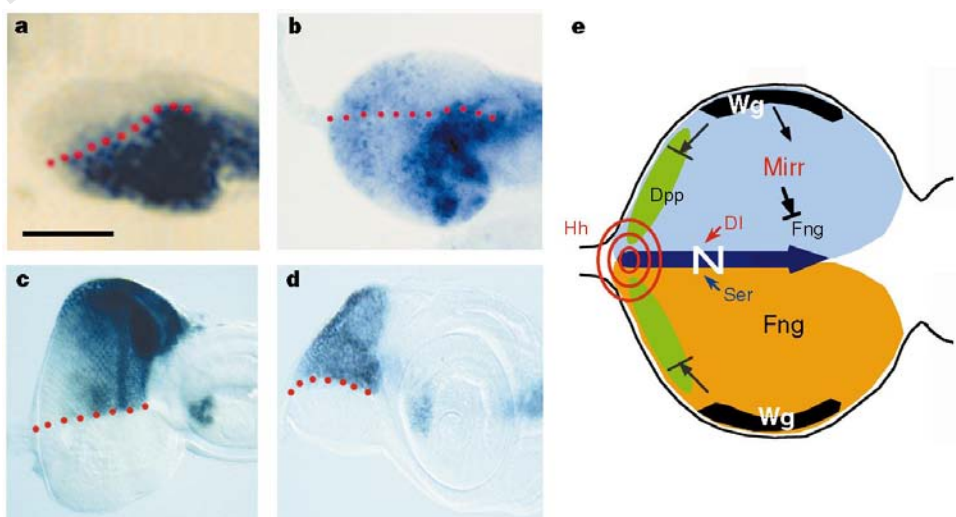




**Figure 4** Failure of eye development by ubiquitous Fng expression. **a–c**, Scanning electron microscopy of adult eyes. **d–f**, Eye discs stained with anti-Elav antibody. **a, d**, Wild type. **b, c, e, f**, *69B-Gal4/UAS-fng22* grown at 22°C (**b, e**) or 25°C (**c, f**); Fng misexpression disrupts eye development. **g**, Time window for the effects of ubiquitous Fng expression. *69B-Gal4/UAS-fng27* embryos were collected at 18°C for 24 h. The start time of egg collection was set as day 0. At different times, the embryos were shifted to 25°C for one day to induce *fng* and then allowed to develop at 18°C to adulthood (experiments 1 to 7). Flies with reduced eyes (smaller than 25% of the normal eye) were scored. Anterior is to the right. Scale bars: **a–c**, 100 µm; **d–f**, 50 µm.



**Figure 5** Effects of ubiquitous Fng expression on Notch (N) signalling proteins and *Eq-3*. **a–c**, *69B-Gal4/UAS-fng27* larvae treated as in experiment 4 of Fig. 4g. At the end of the temperature shift, eye discs were stained with anti-N (**a**), anti-DI (**b**) or anti-Ser (**c**) antibody. Dotted line indicates the equator. **d, e**, Expression of *Eq-3*. Third-instar discs were labelled with anti-β-galactosidase (green) and anti-Bar (red) antibodies<sup>28</sup> to detect *Eq-3* and photoreceptors R1/6, respectively. LacZ expression in the *Eq-3 UAS-fng22/TM6* control eye coincides with the equator (**d**). LacZ expression remains in the dorsal branch (arrow) but is not detected in the equator (dashed line) of the *Eq-3 UAS-fng22/69B-Gal4* eye (**e**). Most cells in the red patch (arrowhead) are Bar-expressing non-neuronal cells. Dotted lines indicate the boundary of the disc. Dorsal is up and anterior is to the right. Scale bars: **a–c**, 5 µm; **d, e**, 50 µm.



**Figure 6** Repression of Fng by Mirr. **a**, *fng* expression in normal first-instar eye as shown in Fig. 1a. **b**, Ventral *fng* expression is lost in the eye disc from *dpp-Gal4/UAS-mirr* flies grown at 25°C. **c**, Dorsal Mirr expression in a third-instar disc of *mirr<sup>B1-12</sup>/TM3* enhancer trap line. **d**, Dorsal Mirr expression is maintained in *mirr<sup>B1-12</sup> 69B-Gal4/UAS-fng27* flies grown at 25°C. **e**, A model for the role of Fng. Mirr can antagonize Fng expression, so providing a mechanism for the ventral

specificity of Fng. Ser and DI activate Notch (N) at the equator. Intersection of equatorial Notch signalling and an unknown factor at the posterior margin (red circles) is critical for eye morphogenesis, as the eye fails to develop in the absence of an equatorial boundary (Fig. 4). Wingless (Wg) suppresses Dpp to prevent the formation of ectopic furrow from the lateral margins<sup>29</sup> and is important in the regulation of Mirr expression<sup>18</sup> and planar polarity<sup>11,30</sup>.

*fng22* flies were reduced to variable sizes at room temperature but were not affected at 18 °C. At 25 °C, all progeny had either no or greatly reduced eyes (Fig. 4b, c). Eye discs from *69B-Gal4/UAS-fng22* larvae showed a complete lack of or greatly reduced numbers of photoreceptors when stained with anti-Elav antibody, a neuronal marker, indicating that photoreceptors failed to develop (Fig. 4e, f).

We performed temperature-shift experiments to determine which stage of eye development was sensitive to ubiquitous Fng expression. *69B-Gal4/UAS-fng27* eggs were collected and incubated at 18 °C, transferred to 25 °C for one day at different time points, and then allowed to develop at 18 °C to adulthood (Fig. 4g). When the animals were shifted to 25 °C on the fourth day, over 90% of flies had small eyes (experiment 4 in Fig. 4g). Temperature shifts at other times showed no significant effect either on the eye size or on polarity (data not shown). Therefore, the Fng boundary during late first-instar to mid second-instar stages is critical for eye morphogenesis. When *69B-Gal4/UAS-fng27* larvae were shifted to 25 °C for one day during the critical time, the equatorial expression of Notch and Df (Fig. 1d, g) in early eye discs was replaced by ubiquitous distribution (Fig. 5a, b). Ventral expression of Ser (Fig. 1h) was either lost or mislocalized (Fig. 5c). The *Eq-3 lacZ* reporter is expressed along the equator, posterior to the morphogenetic furrow. Anterior to the furrow, the equatorial expression of *Eq-3* bifurcates dorsoventrally, but mostly in the dorsal region (Fig. 5d). In third-instar discs from *69B-Gal4/UAS-fng22* larvae, *Eq-3* expression at the equator region was either absent or greatly reduced (Fig. 5e). Therefore, the Fng boundary is required for proper equatorial expression of *Eq-3* as well as of Notch, Df and Ser.

Other domain-specific genes may contribute to the formation of proper polarity in the eye. In contrast to ventral-specific expression of Fng, a homeobox protein, Mirror (Mirr), is expressed in the dorsal side of the eye<sup>15–17</sup> and is controlled by Wingless (Wg)<sup>18</sup>. We examined whether expression of Mirr in the ventral region might alter the pattern of Fng expression. Misexpression of Mirr in both DV domains by *dpp-Gal4/UAS-mirr* greatly reduced the Fng level in the ventral region (Fig. 6a, b). However, ubiquitous Fng expression by *69B-Gal4/UAS-fng27* caused reductions in the size of the eye disc but no significant changes in Mirr expression (Fig. 6c, d). This suggests that Mirr prevents Fng expression in the dorsal region and that the Fng boundary may be established and/or maintained by the antagonistic action of Mirr (Fig. 6e).

This study indicates that Fng is crucial in establishing the DV boundary in early development of the *Drosophila* eye. The Fng boundary results in the activation of Notch signalling at the equator, which in turn may facilitate proliferation when the equator intersects with the posterior margin of the disc. It is noteworthy that Hedgehog (Hh) is expressed at the posterior disc margin and is critical for the initiation of differentiation through interaction with Decapentaplegic (Dpp)<sup>19–23</sup>. Hh or an unidentified factor at the posterior disc margin might be involved in interacting with equatorial Notch to organize eye development (Fig. 6e). □

## Methods

**Fly stocks.** *69B-Gal4*, *dpp<sup>bkl</sup>-Gal4*, *UAS-lacZ* and *dpp-lacZ* reporter *H1.1* were provided by Bloomington Stock Center. *fng* alleles and *UAS-fng* strains<sup>5</sup> were obtained from J. Kim. A *P-lacW* enhancer detector line, *Eq-3*, was initially isolated by Q. Sun. It shows equatorial *P[w<sup>+</sup>]* expression in the eye, similar to *Eq-1* and *Eq-2* (ref. 24). *mirr<sup>B1-12</sup>* was described in ref. 16.

**Analysis of *fng<sup>+</sup>* or *fng<sup>+</sup>* clones.** Two *fng* null alleles, *fng<sup>13</sup>* and *fng<sup>80</sup>*, were used to generate mosaic clones in adult eyes, and the latter was also used for clonal analysis in larval eyes. *y w*; *P[mini-w<sup>+</sup>: vg<sup>en</sup>]*; *mwh jv fng<sup>13</sup>/L14(SM5 + TM6b)* males were crossed to *w<sup>1118</sup>*; *P[mini-w<sup>+</sup> hs-πM]75C*, *P[ry<sup>+</sup>: hs-neo: FRT]80B* females<sup>12</sup>. First-instar larvae from this cross were incubated at 37 °C for 30 min to induce FLP-mediated recombination. For γ-ray-induced mitotic recombination, *w<sup>1118</sup>*; *fng<sup>80</sup>* flies were crossed to *w<sup>1118</sup>*; *P[w<sup>+</sup>]33* (ref. 25). First-instar larval progeny were irradiated with 1,000–1,200 rad. Adult mosaic eyes were embedded in Epon for sectioning. Eye discs were stained for immunofluor-

escent images as described below. To generate *fng<sup>+</sup>* clones, first-instar larvae from the cross between *UAS-lacZ*; *UAS-fng22* males and *y w* *P[Actin > CD2 > Gal4; w<sup>+</sup>]*; *hsFlp*, *MKRS/TM6 Tb* females (provided by K. Kozopas) were treated at 38 °C for 1 h and cultured at room temperature until second instar.

**Scanning electron microscopy, immunocytochemistry and mRNA in situ hybridization.** For scanning electron microscopy, adult eyes were dehydrated, critical-point dried and examined by JEOL 6100 electron microscope. For immunocytochemistry with horseradish peroxidase (HRP)-conjugated secondary antibodies, eye discs were fixed in periodate–lysine–paraformaldehyde fixative and stained as described<sup>26</sup>. Immunofluorescent staining was done as described<sup>27</sup>. Eye discs containing *fng<sup>+</sup>* clones were stained with X-gal before antibody staining<sup>13</sup>. Anti-Bar (1:50), anti-Elav (1:10), anti-Df (1:1,000), anti-Notch (1:1,000), anti-Ser (1:10), anti-Myc (Molecular Probe, 1:10) and anti-β-galactosidase (Promega; 1:250) were primary antibodies used in this study. Secondary antibodies were anti-mouse-HRP, anti-rabbit-CY3, anti-mouse-fluorescein isothiocyanate (FITC; Jackson Laboratories) and anti-rabbit-HRP (Biorad). *fng* complementary DNA was linearized and amplified by polymerase chain reaction using one primer to make digoxigenin-labelled single-stranded DNA probe for *in situ* mRNA hybridization<sup>26</sup>. Transgenic lines containing *UAS-mirr* were made by subcloning of *mirr* cDNA<sup>16</sup> into pUAST vector<sup>14</sup> and by germ-line transformation (B. T. Kehl and K.-W.C., unpublished results).

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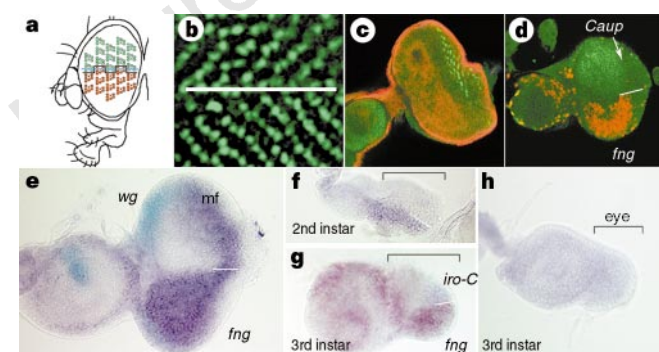
## A dorsal/ventral boundary established by Notch controls growth and polarity in the *Drosophila* eye

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In the *Drosophila* compound eye the dorsal and ventral fields of eye units (ommatidia) meet along the dorsoventral midline, forming a line of mirror image symmetry called the equator<sup>1</sup>. The molecular mechanism establishing the equator is not fully understood, but it involves the transcription factors<sup>2</sup> encoded by the *Iroquois* gene complex<sup>3</sup>. The *Iroquois* genes are expressed in the dorsal half of the eye<sup>2</sup> and here we show that they regulate the expression of the secreted molecule Fringe. A boundary between *fringe*-expressing and *fringe*-non-expressing cells is essential, from the time of the second larval instar, for eye growth and formation of the equator. Boundaries of *fringe* expression determine where the transmembrane receptor Notch is activated<sup>4,5</sup>. We find that Notch is activated at the dorsoventral midline, where it is required to promote growth and set up the axis of mirror symmetry. As boundaries of *fringe* expression and Notch activa-

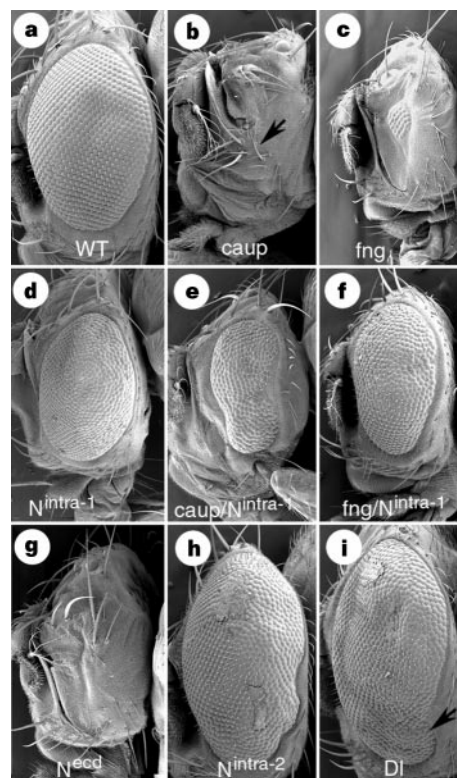


**Figure 1** Dorsoventral organization of the eye disc. **a**, Representation of a left eye showing dorsal (green) and ventral (red) ommatidia, the dorsoventral midline (pale blue straight line) and the equator (black zigzagging line). **b**, Expression of the transcription factors BarH1/H2 in the photoreceptor cells R1 and R6 (green). The ommatidia rotate in opposite directions above and below the midline<sup>1</sup> (white line). **c**, *eyeless-Gal4/UAS-β-galactosidase* (red) and Spalt (green) expression in mid-third-instar eye disc. **d**, Caupolican (green) and *fng-lacZ* (red) expression in mid-third-instar stage. **e**, **f**, *fng* RNA (purple) and *wingless-LacZ* (*wg*, blue) in early-third- (**e**) and second- (**f**) instar eye discs. mf, morphogenetic furrow. **g**, *araucan-lacZ* (*iro-C*, grey) and *fng* (purple) RNA in *eyeless-Gal4/UAS-Necd* larvae. **h**, Expression of *fng* RNA (grey) in the *eyeless-Gal4/UAS-caupolican* disc. Anterior is to the left. The eye region is indicated by brackets in **f–h**. The disc in **f** is shown at larger magnification than in the other panels.

tion are also important during *Drosophila* wing formation<sup>6</sup> and vertebrate somitogenesis<sup>7–9</sup>, we suggest that these boundaries constitute a general mechanism that directs growth and patterning of large fields of cells.

The establishment of the equator (Fig. 1a, b) depends on dorsoventral coordinates that exist before ommatidial differentiation begins<sup>2,10–13</sup>. The expression of *mirror*<sup>2</sup> and two other members of the *Iroquois* gene complex (*araucan* and *caupolican*) in the eye imaginal disc is restricted to dorsal cells during the second larval instar (data not shown). Later, *Iroquois* expression is restricted to dorsal cells anterior to the morphogenetic furrow (Fig. 1d). We find that *fringe* (*fng*) is expressed during the second larval instar in a ventral domain complementary to that of *Iroquois* expression (Fig. 1d–f). Ectopic expression of *caupolican* represses *fng* expression (Fig. 1c, h), indicating that *fng* may be restricted to the ventral half because it is repressed dorsally by Iroquois proteins.

When either *fng* or its repressor *caupolican* is uniformly expressed early in development using the Gal4 system<sup>14</sup>, the resulting eyes are dramatically reduced in size (Fig. 2a–c), demonstrating the importance of restricted expression of *fng* in promoting eye growth. The failure to form normal-sized eyes is not related to direct effects on cell differentiation, because ectopic expression of *caupolican* or *fng* in and behind the morphogenetic furrow does not affect the growth of the eye or the differentiation of the ommatidia (data not shown). As, in the wing, boundaries of *fng* are involved in the activation of Notch<sup>5</sup>, we wondered whether a dorsal/ventral boundary established by Notch before the onset of ommatidial differentiation



**Figure 2** Consequences of modifying Notch activity in eye development. Adult heads of female wild-type flies and combinations between different UAS lines and *eyeless-Gal4*. **a**, Wild-type (WT). **b**, **c**, Misexpression of *caupolican* (**b**) and *fng* (**c**) results in the formation of very small eyes. Duplication of dorsal bristles in **b** is indicated by an arrow. **d–f**, Misexpression of a strong, ligand-independent activated Notch (*N<sup>intra-1</sup>*, **d**) rescues the formation of the eye when it is expressed simultaneously with UAS-*caupolican* (*caup/N<sup>intra-1</sup>*, **e**) or UAS-*fng* (*fng/N<sup>intra-1</sup>*, **f**). **g**, Misexpression of a dominant-negative Notch (*N<sup>ecd</sup>*) results in the reduction in size or disappearance of the eye. **h**, **i**, Misexpression of a weak *N<sup>intra</sup>* construct (*N<sup>intra-2</sup>*, **h**) or the Notch ligand Delta (*DI*, **i**) results in the formation of large eyes; the enlargement is mainly in the dorsoventral axis (arrow in **i**). Anterior is to the left.



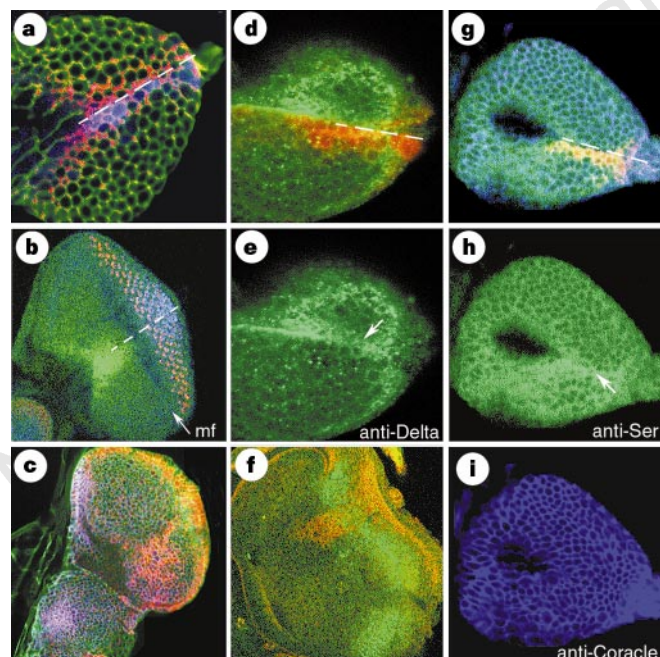
could control growth in the eye. We found that uniform expression of an activated, ligand-independent form of Notch ( $N^{intra}$ )<sup>15</sup> (Fig. 2d–f) rescues the small-eye phenotype caused by uniform expression of *caupolican* or *fng*—the eye is larger, but ommatidia are abnormally arranged. Thus, the phenotypes resulting from misexpression of *caupolican* and *fng* are caused by a failure to activate Notch, indicating that Notch is required for the formation of the eye.

We next investigated the requirement for Notch signalling directly by expressing activated ( $N^{intra}$ )<sup>15</sup> and dominant-negative ( $N^{ecd}$ )<sup>16</sup> forms of Notch uniformly in early discs. Misexpression of  $N^{ecd}$  causes a dramatic reduction in the size of the eye (Fig. 2g) comparable to that caused by misexpression (Fig. 2c) or inhibition (Fig. 2b) of *fng*. However, when  $N^{ecd}$  is misexpressed the expression of *fng* is still restricted to ventral cells (Fig. 1g), indicating that the requirement for Notch is downstream of *fng*. Misexpression of a weak  $N^{intra}$  or of the Notch ligand Delta (DL) has the opposite effect on eye size, resulting in the formation of larger than normal eyes (Fig. 2h, i). The effects of Notch on growth correlate with alterations in the establishment of the dorsoventral midline, as monitored by the restricted expression of the *vestigial* dorsal/ventral boundary enhancer<sup>17</sup>. The gene *vestigial* is not normally expressed in eye discs. However, the dorsal/ventral boundary enhancer construct (*vg d/v*), which contains several sites that bind Suppressor of Hairless, a signal transducer downstream of Notch<sup>17,18</sup>, is expressed in second-instar eye discs in ventral cells along the dorsoventral midline (Fig. 3a). Ectopic expression of  $N^{intra}$  or DL causes the ectopic activation of this

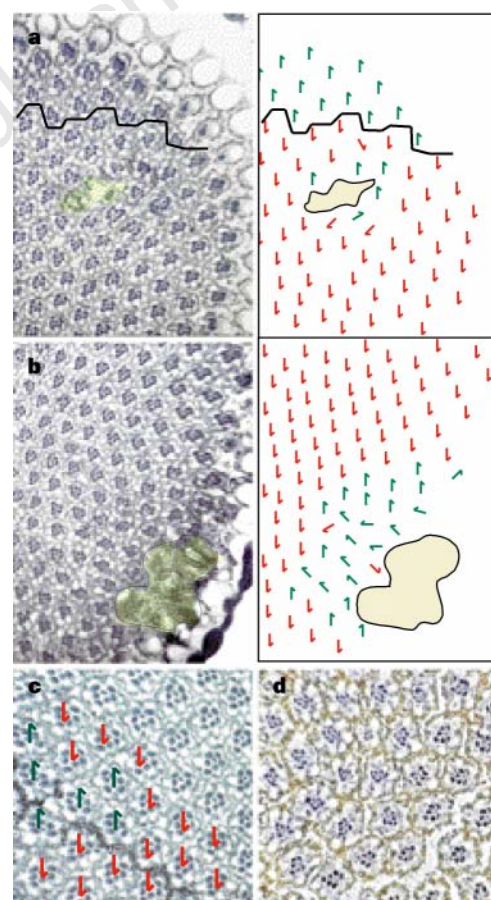
enhancer in many ventral cells (Fig. 3c; data not shown). Conversely, mutations in *Suppressor of Hairless* produce a small-eye phenotype, and the expression of *vg d/v* is lost (data not shown).

We suggest that the effects of modifying Notch activity in early eye discs are the result of perturbations in the establishment of a dorsoventral organizing centre required for eye growth. To determine directly in which cells Notch is activated, we examined the expression of *Enhancer of split-mβ* (*E(spl)mβ*), a gene regulated by Notch<sup>18</sup>. In early eye discs, the expression of *E(spl)mβ* is detected at high levels along the dorsoventral midline (Fig. 3a). Later, this dorsoventral expression is detected only at location anterior to the morphogenetic furrow (Fig. 3b). Two Notch ligands, DL and Serrate (Ser), are also localized at the dorsoventral midline. DL protein accumulates preferentially at the dorsal side (Fig. 3d, e), whereas Ser accumulates at the ventral side (Fig. 3g, h). Maximal expression of Ser in cells that express *fng* also occurs in the wing disc<sup>6</sup>.

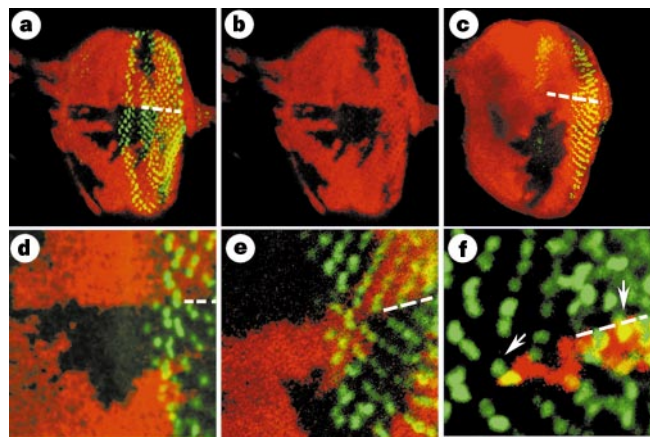
Ommatidial differentiation is not affected when activated forms of Notch or Notch ligands are expressed anterior to the morphogenetic furrow. However, the resulting adult eyes show erratic



**Figure 3** Accumulation of Notch ligands and restriction of Notch activation. **a**, Expression of *E(spl)mβ-CD2* (red) and *vg d/v* (purple) in the second instar along the dorsoventral midline (white dashed line). Cell membranes are labelled by Coracle (green). **b**, Expression of *E(spl)mβ-CD2* (green) in the third instar is anterior to the morphogenetic furrow (mf), aligned with the expression of an equatorial marker (*WR122*, light blue) in differentiating cells. The equator (white dashed line) is visualized by Spalt expression (pink). **c**, Ectopic expression of *vg d/v* (red) in *eyeless-Gal4/UAS-DL* larvae. Orientation of the disc and cell outlines are visualized by *wingless* (purple) and Coracle (green) expression, respectively. **d, e**, Expression of DL (green) and *vg d/v* (red). **f**, Expanded expression of the equatorial marker *WR122* (green) in *eyeless-Gal4/UAS-N<sup>intra</sup>* larvae. Expression of Coracle is in red. **g, h**, Co-localization of Ser (green) and *vg d/v* (pink) in ventral cells abutting the dorsoventral midline. **i**, The distribution of another membrane-associated protein, Coracle, is homogeneous (purple in **i**). Anterior is to the left. The magnification of **a, d, e, g-i** is four times than of the other panels.



**Figure 4** Requirements for *fringe* during eye development. **a, b**, Tangential sections through mosaic eyes carrying *fng<sup>13</sup>/fng<sup>13</sup>* clones near the normal equator (**a**) or in the ventral-most region of the eye (**b**). Clones were visualized by the absence of *white<sup>+</sup>* pigment and are shown in the yellow areas. *fng<sup>13</sup>* clones close to the equatorial region repolarize adjacent ommatidia over a short distance (**a**, green half-arrows below the equator (black line)) and a bulge in the eye and long-range repolarizing effects occur in clones located in more ventral regions (**b**, green half-arrows). **c**, Twin clone (carrying *fng<sup>+</sup>/fng<sup>+</sup>*), recognized as dark *white<sup>+</sup>* pigment, also causes repolarization of adjacent *fng<sup>-</sup>/fng<sup>+</sup>* ommatidia. **d**, Section through an *eyeless-Gal4/UAS-DL* eye, showing disruptions in dorsoventral polarity, visualized as abnormal orientations of photoreceptor rhabdomeres. The polarity of relevant ommatidia in **a-c** is indicated by red (ventral) and green (dorsal) arrows. Note that some ommatidia also have inverted anterioposterior polarity.



**Figure 5** Cell-lineage analysis in the eye disc. **a–f**, *Minute*<sup>+</sup> clones (absence of red) induced 48–72 h after egg-laying and visualized in late-third-instar discs. The expression of Spalt (green, in R3/R4 cells, **a**, **d**, **f**) and Bar-H1/H2 (green, in R1/R6 cells, **c**, **e**) was used to visualize the equator (dotted white line). Note the straight dorsoventral lineage restriction border (**a**, **d**, **e**) crossing the equator. **b** shows the red channel of **a**. Individual ommatidia can be formed by dorsal and ventral cells (**f**, arrows), in agreement with analysis of adult eyes<sup>21–23</sup>.

dorsoventral polarity (Fig. 4d), and the expression of an equatorial marker (WR122)<sup>11</sup> is ubiquitous in the corresponding imaginal disc (Fig. 3f), indicating that Notch may also be involved in the positioning of the equator. This conclusion is reinforced by the effects on ommatidial polarity of novel confrontations between cells that do and do not express *fng*. Homozygous *fng* mutant clones in the ventral half of the eye cause non-autonomous repolarization of wild-type ommatidia over long distances (Fig. 4a, b). Cells carrying two doses of *fng*<sup>+</sup> (*fng*<sup>+</sup>/*fng*<sup>+</sup> twin clones) can also repolarize adjacent *fng*<sup>+</sup>/*fng*<sup>−</sup> tissue (Fig. 4c), indicating that an equator may be formed wherever there is an interaction between cells expressing different levels of *fng*. Furthermore, local increases in cell proliferation are associated with ventral *fng* clones located away from the equator (Fig. 4b), supporting the idea that *fng*<sup>+</sup>/*fng*<sup>−</sup> confrontations create growth-promoting boundaries.

The regulation and function of Notch at the dorsal/ventral boundary in the eye and wing discs appear to be conserved. In the wing, dorsal and ventral cell populations have different identities because *apterous* is expressed in dorsal cells alone, and the populations correspond to two independent lineage compartments<sup>19,20</sup>. Dorsal and ventral identities in the adult eye do not exist<sup>1</sup>, but a cell-lineage restriction boundary appears to separate the dorsal and ventral halves<sup>21–23</sup>. We studied cell lineage in the eye disc by making *Minute*<sup>+</sup> clones, and found a lineage restriction that approximates the position of the equator, where clones have straight borders (Fig. 5a, b, d, e). In contrast, the polar border of the same clones is always wiggly, as are other clone borders in most internal regions of the disc (Fig. 5c). The equator follows a zigzagging line, and therefore does not exactly coincide with the dorsoventral restriction boundary, and photoreceptor cells belonging to ommatidia with opposite chirality can form part of the same clone (Fig. 5f; refs 21–23).

In summary, our results show that a dorsal/ventral organizing centre established by Notch before the onset of retinal differentiation regulates eye growth and polarity. The regulatory interactions described here are similar to those occurring in the wing and haltere discs<sup>6</sup>, suggesting a common underlying mechanism for patterning all dorsal discs. □

## Methods

**Drosophila strains.** We used *fng*–*lacZ* (35UZ-1)<sup>4</sup>, *eyeless*–*Gal4* (a gift from U. Walldorf), *Enhancer of split* mβ-CD2 (ref. 24), the equatorial marker WR122–

*lacZ* (ref. 11) and the lines *UAS*–*caupolican* (ref. 2), *UAS*–*fng* (ref. 5), *UAS*–*N<sup>ecd</sup>* (ref. 16), *UAS*–*N<sup>intra-1</sup>*, *UAS*–*N<sup>intra-2</sup>* and *UAS*–*Dl* (ref. 6), where *UAS* is upstream activation sequence. *UAS*–*N<sup>intra-1</sup>* is a full-length intracellular Notch fragment (a gift from M. Haelin), whereas *UAS*–*N<sup>intra-2</sup>* carries an internal deletion and results in weaker Notch activity<sup>15</sup>. All combinations of *eyeless*–*Gal4* and different *UAS* lines were created at 25 °C.

**Generation of mosaics.** Clones of the *fng*<sup>13</sup> allele<sup>4</sup> were induced in first/second-instar larvae of *yw hsp-70-flp; fng<sup>13</sup> FRT80B/tubulinα-lacZ* (*w*<sup>+</sup>) *FRT80B* genotype by 1-h heat-shock at 38 °C. Adult eyes were processed for sectioning and analysis as described<sup>21</sup>. Cell lineage *Minute*<sup>+</sup> clones were induced by Flipase-mediated mitotic recombination in *yw hsp-70-flp; FRT42D arm-lacZ M(2)53/FRT42D hsp-70-πMyc* larvae. Clones were labelled by the absence of β-galactosidase expression.

**In situ hybridization and immunocytochemistry.** X-gal staining, whole-mount *in situ* hybridization and antibody staining were done as described<sup>25</sup>. The antibodies used were: rabbit anti-β-galactosidase (Cappel), mouse anti-CD2 (Serotech), rabbit anti-BarH1/H2 (a gift from S. Higashijima), rat anti-Caupolican (a gift from J. Modolell and P. Aroca), rat anti-Spalt (a gift from R. Barrio), rabbit anti-Ser, mouse anti-Dl and guinea-pig anti-Coracle (described in ref. 24). Secondary fluorescent antibodies were from Jackson.

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# Energy transduction in the $F_1$ motor of ATP synthase

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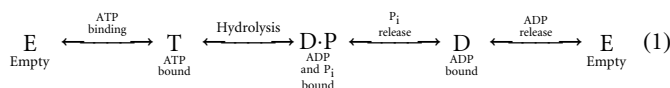
ATP synthase is the universal enzyme that manufactures ATP from ADP and phosphate by using the energy derived from a transmembrane protonmotive gradient. It can also reverse itself and hydrolyse ATP to pump protons against an electrochemical gradient. ATP synthase carries out both its synthetic and hydrolytic cycles by a rotary mechanism<sup>1-4</sup>. This has been confirmed in the direction of hydrolysis<sup>5,6</sup> after isolation of the soluble  $F_1$  portion of the protein and visualization of the actual rotation of the central 'shaft' of the enzyme with respect to the rest of the molecule, making ATP synthase the world's smallest rotary engine. Here we present a model for this engine that accounts for its mechanochemical behaviour in both the hydrolysing and synthesizing directions. We conclude that the  $F_1$  motor achieves its high mechanical torque and almost 100% efficiency because it converts the free energy of ATP binding into elastic strain, which is then released by a coordinated kinetic and tightly coupled conformational mechanism to create a rotary torque.

The general structure of ATP synthase is shown in Fig. 1. It consists of two portions: a soluble component,  $F_1$ , containing the catalytic sites, and a membrane-spanning component,  $F_0$ , comprising the proton channel. The entire  $F_0F_1$  structure is arranged as a counter-rotating 'rotor' and 'stator' assembly. The stator portion consists of the catalytic sites contained in the  $\alpha_3\beta_3$  hexamer, together with subunits  $a$ ,  $b_2$  and  $\delta$ . The rotor consists of 9-12  $c$ -subunits arranged in a ring and connected to the  $\gamma$ - and  $\epsilon$ -subunits that form the central 'shaft'. The mechanism of protonmotive force transduction has been discussed elsewhere<sup>7</sup>; we now focus on the mechanochemistry of  $F_1$ , which synthesizes ATP when the  $\gamma$ - $\epsilon$  shaft is turned clockwise (viewed from the membrane) by the protonmotive force, and which rotates counterclockwise to pump protons when hydrolysing ATP.

Any mechanism proposed to explain the experimental results is strongly constrained by thermodynamic, kinetic, structural and mechanical considerations. In the hydrolysis direction, it must reproduce the large torque and almost 100% mechanical efficiency<sup>5,6</sup>; in the synthesis direction, ATP must be produced at the measured rate when driven by torques generated by the protonmotive force in  $F_0$ . Kinetic constants and free energies must correspond to measured values. A model must also be geometrically compatible with the detailed structural information now available.

We constructed the model in three steps. First, kinematic motions of the  $F_1$  subunits were analysed to determine the conformational changes that drive rotation of the  $\gamma$ -subunit. From this, we constructed a mathematical model describing the principal mechanical and electrostatic interactions between the subunits. Finally, the reaction sequences were incorporated to create a complete mechanochemical model. The outline of this model can be summarized as follows.

The reactivity of a catalytic site depends on its local conformation and composition. The unique feature of  $F_1$  is that the reactivity of each site depends on the states of the other two sites, and the position of  $\gamma$ . Each of the three catalytic sites,  $\beta_1$ ,  $\beta_2$  and  $\beta_3$ , passes through at least four chemical states:



We denote by  $\beta_i$  the chemical state (E, T, D-P, D) of the  $i$ th  $\beta$ -subunit, so that  $\alpha_3\beta_3$  has a total of  $4^3 = 64$  possible kinetic states. The collection of these states can be visualized as the integer points in a  $4 \times 4 \times 4$  cube (actually, a three-dimensional torus, because when the state exits one face of the cube, it re-enters the opposite face). The progression of the system through this chemical-state space depends on the angular position of the  $\gamma$ -subunit, which we denote by  $\theta$ . The kinetics can be described by a set of equations (that is, a Markov model) of the form

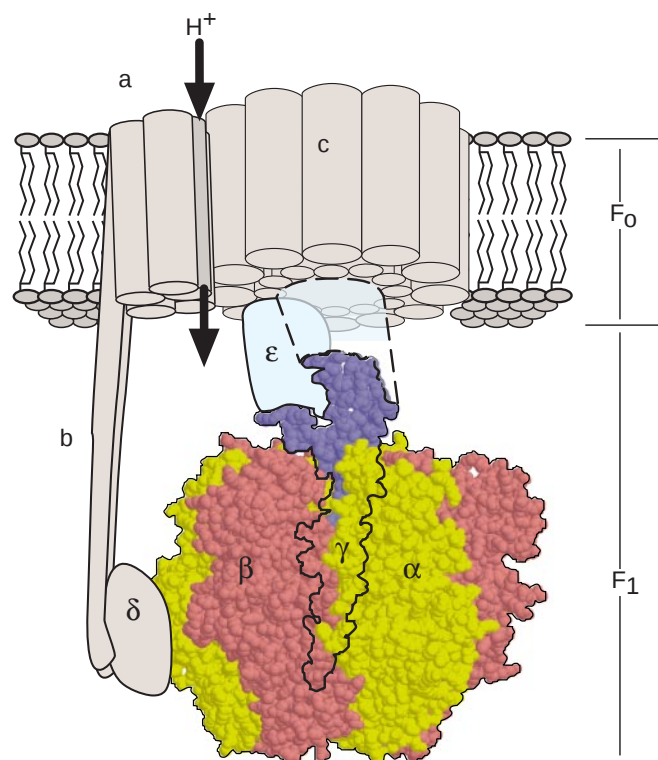
$$d\beta_i/dt = K(\theta, \beta_{i-1}, \beta_{i+1}) \times \beta_i \quad (2)$$

where  $i = 1, 2, 3$  and where  $K$  is the matrix of kinetic transition rates for a catalytic site, which depends on the angular position of  $\gamma$  and the occupancy of the other sites. Thus the state of  $F_1$  is specified by its rotational position and its kinetic state:  $(\theta, \beta_1, \beta_2, \beta_3)$ .

The motion of  $\gamma$  is described by balancing the torques on the rotating  $\gamma$ -subunit:

$$\underbrace{\zeta \frac{d\theta}{dt}}_{\text{Viscous torque on } \gamma} = \underbrace{\tau(\theta, \beta_1, \beta_2, \beta_3)}_{\text{Torque between } \gamma \text{ and } \alpha_3\beta_3} - \underbrace{T_L}_{\text{Load torque}} + \underbrace{\mathcal{T}_B(t)}_{\text{Brownian torque}} \quad (3)$$

The viscous load on the rotating  $\gamma$ -subunit is contained in the drage coefficient,  $\zeta$ .  $T_L$  represents any additional load torque (such as from a laser trap).  $\mathcal{T}_B(t)$  is the brownian torque due to thermal fluctuations. The driving torques exerted on  $\gamma$  by the  $\beta$ -subunits can be expressed as the gradient of an elastic potential:  $\tau(\theta, \beta_1, \beta_2, \beta_3) = -\partial V(\theta, \beta_1, \beta_2, \beta_3)/\partial\theta$ . This potential is derived from the geometry



**Figure 1** Structure of ATP synthase. It has a transmembrane portion,  $F_0$ , consisting of the stoichiometric subunits  $c_{12}ab_2$ , and a soluble portion,  $F_1$ , which consists of subunits  $\alpha_3\beta_3\gamma\epsilon\delta$ . Functionally, the protein behaves as a 'rotor' ( $c_{12}\gamma\epsilon$ ) and a 'stator' ( $ab_2\delta\alpha_3\beta_3$ ), which are thought to counter-rotate during hydrolysis and synthesis. The three catalytic sites are located at the  $\alpha\beta$  interfaces, and the proton channel is at the  $c$ - $a$  interface. The dashed outline represents the fragment of  $\gamma$  that has not been resolved. In recent hydrolysis experiments<sup>5,6</sup>, the  $\alpha_3\beta_3$  subunit was attached to a coverslip and a fluorescently tagged actin filament was attached to the  $\gamma$ -subunit so that rotation of  $\gamma$  could be observed; here, only subunits  $\alpha_3\beta_3\gamma$  were present.



and elastic properties of the mechanical model for  $F_1$ . We constructed this mechanical model by the following procedure.

First, we analysed the kinematic motions of  $F_1$ . To do this, we imported the coordinates for  $\alpha_3\beta_3\gamma$  from the Protein Data Bank into the molecular structure program Rasmol<sup>1,8</sup>. According to Boyer's binding change mechanism, a  $2\pi/3$  rotation of  $\gamma$  corresponds to a  $2\pi/3$  advance in the kinetic states of the three catalytic sites and a  $2\pi/3$  advance of the conformational asymmetry of the  $\alpha_3\beta_3$  hexamer. This allows us to derive the coordinates of  $F_1$  for  $\theta = 2\pi/3$  from those at  $\theta = 0$ . For angles between 0 and  $2\pi/3$ , the coordinates of  $F_1$  are calculated by interpolation in a cylindrical coordinate system. From this we created stereo animations of the complete motion of  $F_1$  through the hydrolysis cycle (see Supplementary Information). Although the interpolated positions obey all steric constraints, they are probably not exact; nevertheless, they do give an idea of the overall motion of the subunits.

Next, we placed a local coordinate system on each subunit so that we could determine quantitatively the subunit's sequence of conformations. A study of the Protein Motions Database shows that most conformational changes can be resolved into hinge and shear motions<sup>9,10</sup>. The principal motion of the  $\beta$ -subunits consists of a hinge motion that bends the top and bottom segments towards one another by about  $30^\circ$  (Fig. 2), and two much smaller motions which we ignore: a radial shear motion and rotation in the  $\theta$  direction. The  $\alpha$ -subunits undergo very little change in shape.

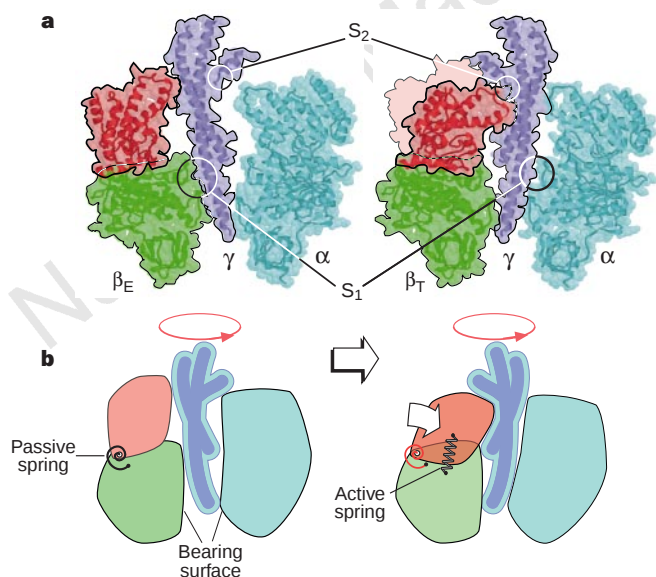
To see how the vertical bending motion is converted into a rotary torque on the  $\gamma$ -subunit, we took horizontal 'serial sections' through the  $\alpha_3\beta_3\gamma$  assembly to determine the cross-sectional shapes of  $\gamma$  and the central channel in  $\alpha_3\beta_3$  into which  $\gamma$  fits. Combining this information, we deduced that the curved and asymmetric  $\gamma$ -subunit is driven by the hinge motion of the

$\beta$ -subunits in a fashion analogous to a hand on the crank of a car jack (see Supplementary Information).

We now make an important assumption about how the free energy from ATP hydrolysis is converted into the hinge-bending motion of  $\beta$ : the energy to bend a  $\beta$ -subunit is conferred on the catalytic site at the nucleotide-binding step in equation (1); that is, the rapid sequential bonding of the nucleotide to the catalytic site is converted into an elastic strain energy of  $\sim 24 k_B T$  ( $14 \text{ kcal mol}^{-1}$ ) centred at the catalytic site. Binding may proceed in several stages: for example, weak binding, followed by a rapid thermal 'zippering' of (mostly hydrogen) bonds—primarily to the phosphate groups. However, for simplicity we treat the binding as a single kinetic step in equation (1). This strain energy tends to bend the  $\beta$ -subunit, and the bending stress is converted by the protein's geometry into a rotary torque on the  $\gamma$ -subunit and to stresses on the other catalytic sites (Fig. 2).

Viewed as an elastic body, the  $\alpha_3\beta_3$  hexamer is complicated, and certainly not isotropic. However, we can construct a simplified mechanical equivalent that captures its principal motions<sup>11,12</sup>. In this way, the complicated elastic behaviour of  $F_1$  can be approximated by an assembly of rigid elements connected by springs (Fig. 2b).

We treat the  $\alpha_3\beta_3$  hexamer as an elastic body with a pre-stress arising from the free energy of assembly. First, we model the intrinsic elasticity of the  $\beta$ -subunits as passive elastic elements at the hinge points. Second, we represent the active stress created by nucleotide binding as much stronger elastic elements that are activated upon nucleotide binding. Third, by assigning reasonable elastic constants to these elements (Box 1), we can compute elastic potential energies based on the simplified molecular structure shown in Fig. 2, one potential curve for each of the 64 kinetic



**Figure 2** Conformation changes in the  $\beta$ -subunit. **a**, Side-view cross-section showing the conformational changes of the  $\beta$ -subunit in the empty (E) and ATP-bound (T) states, and the corresponding rotation of the  $\gamma$ -subunit as deduced from an analysis of the PDB coordinates (see Supplementary Information for procedures and animation moves). The two switch regions<sup>14</sup>, denoted by  $S_1$  and  $S_2$ , control nucleotide binding and phosphate release, respectively, at each catalytic site. **b**, Mechanical model used to compute the elastic potential that drive the rotation of the  $\gamma$ -subunit (described in Supplementary Information). The hinge-bending motion of  $\beta$  rotates the upper portion by  $\sim 30^\circ$ . This is converted into a rotary torque on  $\gamma$  by the asymmetric bend in  $\gamma$ . The torque on  $\gamma$  is contributed by two  $\beta$ -subunits at a time, because each  $\beta$  has two free-energy drops during each hydrolysis cycle (Fig. 3a).

## Box 1 Multisite reaction rates

Multisite reaction rates are constructed from the unisite rates by taking into account the effects of coupling of each catalytic site to the other sites, the two switches on the  $\gamma$ -subunit, and the elastic energy of each site. The elastic constants of the active and passive springs refer to Fig. 2.

Construction of the multisite reaction rate  $k^M$  from the unisite reaction rate  $k^U$ :

$$k_{\text{forward}}^M(\theta, \beta_{i-1}, \beta_{i+1}) = k_{\text{forward}}^U \times f(\beta_{i-1}, \beta_{i+1}) \times g(\theta) \times \exp\left(\frac{\lambda \times \Delta E(\theta)}{k_B T}\right)$$

$$k_{\text{backward}}^M(\theta, \beta_{i-1}, \beta_{i+1}) = k_{\text{backward}}^U \times f(\beta_{i-1}, \beta_{i+1}) \times g(\theta) \times \exp\left(\frac{-(1-\lambda) \times \Delta E(\theta)}{k_B T}\right)$$

$f(\beta_{i-1}, \beta_{i+1})$  contains the effect of the occupancy of other sites. Binding of ATP on other sites increases the reaction on this site.  $g(\theta)$  models the effect of switches  $S_1$  and  $S_2$ : rotation of  $\gamma$  brings the switch regions on  $\beta$  and  $\gamma$  into proximity, which increases the reaction rate.  $\Delta E(\theta)$  is the elastic energy difference between the two chemical states of  $\beta$ . (see Supplementary Information).

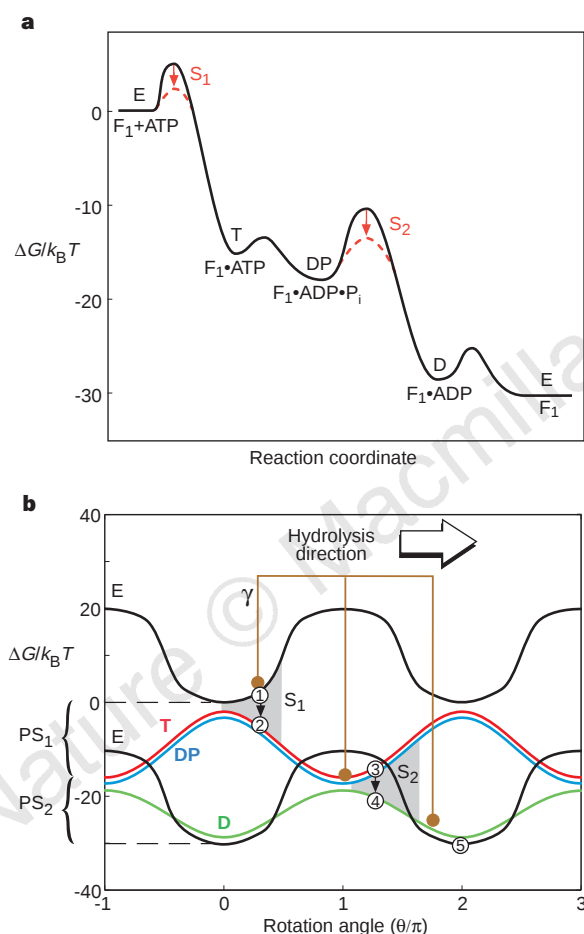
Elastic constants of active and passive springs:

$$k_{\text{active}} = 10 \text{ pN nm}^{-1}, \text{ and } k_{\text{passive}} = 4 \text{ pN nm}^{-1}.$$

The active and passive springs are pre-compressed by 4 nm. During hydrolysis, the additional displacement of these springs is 2 nm.

states. The derivatives of these elastic potentials yield the torques that drive the rotation of  $\gamma$ , as given in equation (3).

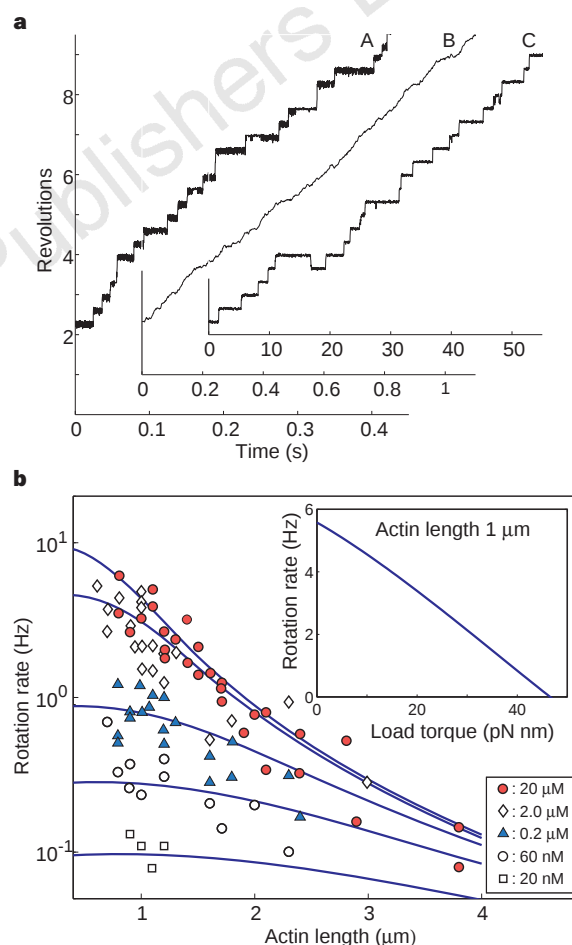
The exact procedure for constructing the potentials is given in the Supplementary Information. Roughly, this consists of measuring the bending motions of a  $\beta$ -subunit by the azimuthal angle,  $\phi$ , between its upper and lower portions. During the course of its conformational change, a  $\beta$ -subunit closes and opens its azimuthal angle by about  $\Delta\phi \approx 30^\circ$ . Trigonometry yields a relationship between the bending angle,  $\phi$ , of a  $\beta$ -subunit and the rotation angle of  $\gamma$ :  $\theta = f(\phi)$ . As the bending angle  $\phi$  closes,  $\beta$  pushes on the off-axis bowed segment of  $\gamma$  (Fig. 2). In this way, a rotary torque in the  $\theta$  direction is generated by the geometric propagation of stress from the catalytic site (the active spring) to  $\gamma$ . As each of the subunits cycles through its conformational range in sequence, a continuous rotational torque is exerted on  $\gamma$ .



**Figure 3** Energy profiles. The free energy of  $F_1$  during its hydrolysis cycle depends on the chemical state of each  $\beta$ -subunit, represented by  $\beta_i = (E, T, DP, D)$ , and the angular coordinate,  $\theta$ , of the  $\gamma$ -subunit:  $\Delta G(\theta, \beta_1, \beta_2, \beta_3)$ . **a**, Projection of the free energy onto the reaction coordinate of a single  $\beta$ -subunit. The free-energy drop proceeds in 4 stages (see equation (1)). The free-energy drops are computed from unisite reaction rates (the free-energy barriers are shown schematically). The transition  $E \rightarrow T$  (nucleotide binding) is activated by switch  $S_1$ ; the transition  $DP \rightarrow D$  (phosphate release) is activated by switch  $S_2$ . **b**, Free-energy profile of the interaction between the  $\gamma$ -subunit and a single  $\beta$ -subunit, showing the 4 elastic periodic potentials along which the system is constrained to move. Numbers trace the trajectory of a catalytic site through one cycle of hydrolysis, which generates two power strokes. The primary power stroke ( $PS_1$ ) occurs upon binding of nucleotide, which drives the bending of the  $\beta$ -subunit. During this bending, the elastic stress turns  $\gamma$  and compresses the passive elastic element. The secondary power stroke ( $PS_2$ ) is triggered by the release of phosphate, causing the elastic energy stored from the primary power stroke to be liberated during the first power stroke of the next site in the sequence.

The unisite (that is, at substoichiometric ATP concentration) rate constants for the transitions given in equation (1) have been measured<sup>13</sup>. But, at steady state the reactions at the three catalytic sites are coordinated in two ways. First, the strain induced by nucleotide binding at each site is communicated to the other two sites. Second, there are at least two 'switch points' on  $\gamma$  that interact with specific sites on the  $\alpha_3\beta_3$  hexamer<sup>14</sup>. Thus, the activation energy barriers for the reaction at each site are influenced by the state of the other two sites and the angular position of the  $\gamma$ -subunit.

Figure 3a shows the free-energy levels of a  $\beta$ -subunit as the system evolves through the 64 possible chemical states. Note that, as measured externally, only part of the free energy of ATP is released to the surroundings upon binding: the remainder is stored internally as elastic deformation and released when phosphate is released. The switch denoted by  $S_1$  in Figs 2 and 3 is located at  $\gamma$ Gln 269 and



**Figure 4** Model predictions. **a**, Simulated rotational trajectories of the  $\gamma$ -subunit relative to  $\alpha_3\beta_3$  at three different ATP concentrations and viscous loads. Trajectory A shows the rotation of  $\gamma$  when the ambient ATP concentration is 2 mM. The motor stochastically steps in increments of  $2\pi/3$ , and the rate is limited by ADP release. In trajectories B and C, a drag coefficient equivalent to an actin filament 1  $\mu$ m long was imposed on the  $\gamma$  subunit<sup>5,19</sup>. Trajectory B corresponds to an ATP concentration of 2 mM. Here the viscous damping of the actin filament obscures the stepping behaviour. Trajectory C shows that the stepping behaviour reappears, even with the drag of the actin filament, when ATP drops to 20 nM. In this situation, hydrolysis is limited by diffusion of ATP to a catalytic site. Note that rare backward steps occur when nucleotide occasionally binds to the 'wrong' site. The average velocities are as measured in ref. 19. **b**, The rotation rate of  $\gamma$  when subjected to viscous loads from a long actin filament. Data points are from ref. 19. Solid lines are computed from the model, and fit the data at all loads and ATP concentrations. If an additional static load is imposed on a 1- $\mu$ m actin filament from, say, a laser trap, the predicted load-velocity relation is shown in the inset.

$\beta$ Thr 304 (*Escherichia coli* sequence numbering); it activates binding of nucleotide successively to each catalytic site. The switch at  $\gamma$ Arg 242 and  $\beta$ Glu 381 of the conserved DELSEED sequence in the  $\beta$ -subunit ( $S_2$  in Figs 2, 3) activates release of phosphate at each site. The interactions between the  $\gamma$ - and the  $\beta$ -subunits at the switch regions are electrostatic and are communicated to the catalytic sites to produce entropic and steric effects that do not involve appreciable changes in free energy.

From the configurational geometry and elastic constants, we can compute the sequence of elastic potentials the  $\gamma$ -subunit experiences from a  $\beta$ -subunit as it undergoes its cycle of nucleotide binding, hydrolysis and product release (Fig. 3b). The elastic energy curves of the other two  $\beta$ -subunits are identical to those shown, but shifted by  $2\pi/3$  and  $-2\pi/3$  respectively. The potential experienced by  $\gamma$  from the three driving  $\beta$ -subunits is the sum of these three sets of potentials. In Fig. 3b, this is represented by the three connected points (offset by  $2\pi/3$ ) on one set of potentials. At one site, the cycle proceeds as indicated by the numbers 1–5, starting where the empty site binds ATP. This drops the state from point (1) on E to point (2) on T; this transition is triggered by  $S_1$ . The exact angle,  $\theta$ , at which ATP binds is stochastic; the vertical arrow in Fig. 3b indicates a typical transition point; the binding of nucleotide strains the catalytic site, and release of this stress through the mechanical escapement shown in Fig. 2 rotates the  $\gamma$  shaft from (2)  $\rightarrow$  (3). This is the primary power stroke. Approximately  $180^\circ$  opposite  $S_1$  on  $\gamma$  is the second switch  $S_2$ , which triggers the release of phosphate (the transition from point (3) on DP to point (4) on D in Fig. 3b), initiating the secondary power stroke. The energy drop from (4)  $\rightarrow$  (5) on the D curve in Fig. 3b releases the elastic strain stored during the primary power stroke. This takes place during the primary power stroke of the next site. Thus, each  $2\pi/3$  rotation of  $\gamma$  is driven by two  $\beta$ s: the binding of nucleotide to one  $\beta$  and the release of elastic strain from the previous  $\beta$  when phosphate is released.

All that remains to complete the model is to specify the chemical rates,  $K(\theta, s)$ , in equation (2), which control the transition between the 64 different elastic potentials. The transition rates are determined by the free energy difference and the energy barrier between adjacent states. As already discussed and summarized in Box 1, the activation energy barrier of a catalytic site is affected by the chemical state of the other two sites and the interaction with the two switch regions on  $\gamma$  (see Supplementary Information for a complete description of the mathematical model and its numerical simulation). The results of these computations are summarized in Fig. 4.

Figure 4a shows typical trajectories for various ATP concentrations and viscous loads. At saturated ATP concentrations (2 mM) and with no additional viscous load, the motor rates in steps of  $2\pi/3$ , consuming one ATP per step (curve A). The rate-limiting step under these conditions is the release of ADP<sup>15</sup>. At low ATP concentrations, and with a large viscous load imposed by the actin filament attached to the  $\gamma$ -subunit<sup>5,6</sup>, the motor also behaves as a stepper (curve C in Fig. 4a). In this situation, the motor is limited by the diffusion of ATP to the catalytic sites<sup>16</sup>. At high ATP concentrations and loads, the step-like progression of the motor is obscured by the viscous drag (curve B in Fig. 4a). At low ATP concentrations, the motor occasionally executes backward steps when nucleotide binds to the 'incorrect' site (curve C in Fig. 4a). These curves reproduce quantitatively the measurements of Yasuda *et al.*<sup>6</sup>. The mechanical efficiency of the motor—computed as the ratio of the average viscous dissipation per step to the free energy of hydrolysis—is very high, approaching 100% for large viscous loads. This high efficiency is possible because the motor is not a heat engine, but is driven almost completely by elastic strain energy.

Figure 4b shows the expected load–velocity relation, together with the velocity as a function of the length of an attached actin filament<sup>5,6</sup>. The motor can generate torques of more than 40 pN nm. The mechanical escapement allows the motor to release the strain

energy conferred by nucleotide binding in two stages. The primary power stroke begins immediately upon nucleotide binding. This power stroke performs two tasks: it drives the  $\gamma$ -subunit in a full  $2\pi/3$  rotation, and it also compresses the passive elastic element, storing nearly half of the elastic energy of binding. This elastic energy is released in a secondary power stroke that assists the next hydrolysis site during its primary power stroke. This two-stage release of stored energy smoothes the output torque and contributes to the high mechanical efficiency of the motor.

The model has three other predictions (see Supplementary Information): (1) it correctly predicts the measured rates of ATP synthesis when driven in reverse by a torque corresponding to that generated in  $F_0$  (refs 2, 17); (2) the proton flux through  $F_0$  coupled to the rotation of  $\gamma$  is blocked by small concentrations of ADP but then increases under normal synthesis conditions, in accordance with results from 'slip' experiments<sup>18</sup>; and (3) the average occupancy of the catalytic sites as a function of ATP concentration agrees well with experiments<sup>13</sup>.

In summary, the model contained in equations (1)–(3) can quantitatively explain the principal features of the  $F_1$  motor. The motor rotates in steps of  $2\pi/3$ , generating up to 45 pN nm of torque and consuming, on average, a single ATP per step, with a mechanical efficiency approaching 100%. To perform in this way, the motor cannot operate as a heat engine but converts the free energy of nucleotide binding into elastic strain energy. This strain energy drives the rotation of the  $\gamma$ -subunit through a tightly coupled mechanical mechanism, which is closely coordinated with the kinetic transitions. The general features of energy transduction in  $F_1$  may have implications for other protein motors driven by nucleotide hydrolysis.  $\square$

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**Supplementary information** is available on Nature's World Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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# Three-dimensional structure of the plant photosystem II reaction centre at 8 Å resolution

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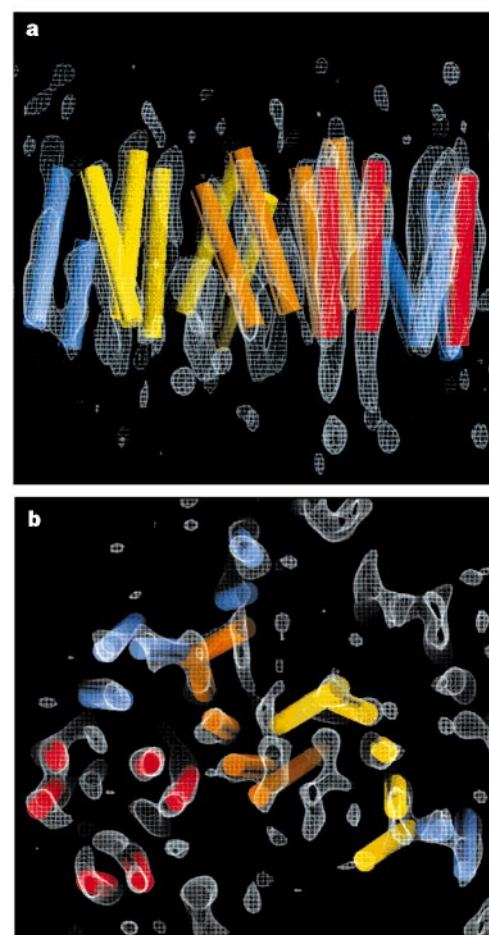
Photosystem II is a multisubunit enzyme complex involved in plant photosynthesis. It uses solar energy to catalyse the breakdown of water to reducing equivalents and molecular oxygen<sup>1</sup>. Native photosystem II comprises more than 25 different subunits, and has a relative molecular mass of more than 600K. Here we report the three-dimensional structure of a photosystem II sub-complex, containing the proteins D1, D2, CP47 and cytochrome *b*-559, determined by electron crystallography. This CP47 reaction centre, which has a relative molecular mass of 160K, can perform light-mediated energy and electron-transfer reactions but is unable to oxidize water<sup>2</sup>. The complex contains 23 transmembrane  $\alpha$ -helices, of which 16 have been assigned to the D1, D2 and CP47 proteins. The arrangement of these helices is remarkably similar to that of the helices in the reaction centres of purple bacteria and of plant photosystem I, indicating a common evolutionary origin for these assemblies. The map suggests that redox cofactors in the D1–D2 complex are located in positions analogous to those in the bacterial reaction centre, but the distance between the chlorophylls corresponding to the bacterial 'special pair' is significantly larger.

Electron micrographs of uncontrasted two-dimensional crystals were recorded at  $-182^{\circ}\text{C}$  at tilt angles ranging from  $0^{\circ}$  to  $65^{\circ}$ . Image processing indicated that crystals were ordered up to  $\sim 6\text{ Å}$ . A three-dimensional data set extending to  $8\text{ Å}$  in the membrane plane and to  $\sim 14\text{ Å}$  in the third dimension was collected (Table 1), and a three-dimensional map calculated.

A cross-section of the map (Fig. 1a) shows that the density is concentrated in a slab of  $\sim 45\text{ Å}$ , as expected for the lipid–protein layer of photosystem II (PSII). The top view of a map segment occupied by one subcore monomer (Fig. 1b) indicates 23 elongated, rod-like regions of density separated laterally by about  $10\text{ Å}$ . These regions are characteristic of membrane-spanning  $\alpha$ -helices at this resolution<sup>3,4</sup>. We assign the  $2 \times 5$  helices that are coloured yellow and orange, and which form a roughly S-shaped feature of near two-

fold symmetry, to the D1 and D2 proteins on the basis of predictions that these proteins are structurally similar to the L and M subunits of the purple bacterial reaction centre<sup>5</sup>. An adjacent group of  $3 \times 2$  helices, coloured red in Fig. 1, is assigned to CP47, on the basis of predictions that this protein has six transmembrane helices<sup>6</sup>. The remaining seven transmembrane helices, shown in blue, cannot be specifically assigned at this stage. They probably belong to the cytochrome *b*-559  $\alpha$ - and  $\beta$ -subunits and to the minor PSII proteins, PsbI, PsbK, PsbL, PsbT and PsbW, which have been shown by mass-spectrometry studies to be present in the complex<sup>7</sup>, and which, from their polypeptide sequences, are predicted to span the membrane once<sup>8</sup>.

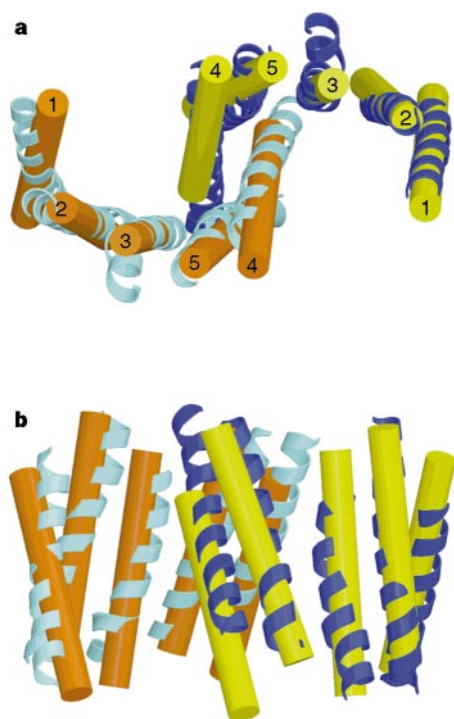
Most of the helices are  $30\text{--}36\text{ Å}$  long, as expected for membrane-spanning helices. The exceptions are two longer rod-like densities in CP47 (see below) and two shorter densities located at either end of the D1–D2 heterodimer, which appear to be only about  $23\text{ Å}$  in length (Fig. 1a) and may not traverse the membrane fully. The densities of the two most highly tilted transmembrane helices in the D1 and D2 proteins were discontinuous, probably because of the limited resolution in the  $z$  direction.



**Figure 1** Three-dimensional map of the monomeric PSII reaction centre complex contoured with a  $1\text{ Å}$  sampling interval at  $2.5\text{ s.d.}$  **a**, Side view (luminal surface below), with cylinders indicating the positions of transmembrane helices. **b**, View from the luminal side, with fitted cylinders showing the 23 membrane-spanning helices in the PSII monomer. The cylinders are colour-coded according to the protein to which they were assigned: D1, yellow; D2, orange; CP47, red; others, blue. Map contours are white. The viewing direction is the opposite of that in the published projection map<sup>15</sup>. When the 11 helices of the PsaA and PsaB proteins are superimposed on the map in such a way that the five C-terminal helices of PsaA and PsaB align with the D2 half of the D1–D2 heterodimer, the six N-terminal helices of PsaA or PsaB can be brought to coincide with the corresponding six helices of CP47 by an in-plane clockwise rotation of  $\sim 15^{\circ}$ .

**Table 1** Electron crystallographic data

|                                   |                                                                                                                                                                                                                                    |
|-----------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Two-dimensional crystals          |                                                                                                                                                                                                                                    |
| Two-sided plane group             | $p22_1$                                                                                                                                                                                                                            |
| Unit-cell parameters              | $a = 168.3\text{ Å}, b = 155.2\text{ Å}, \gamma = 90^{\circ}$                                                                                                                                                                      |
| Thickness (Å)                     | $\sim 75$                                                                                                                                                                                                                          |
| Phase determination from images   |                                                                                                                                                                                                                                    |
| No. of processed images           | 45 ( $0^{\circ}\text{--}10^{\circ}$ : 9; $10^{\circ}\text{--}20^{\circ}$ : 5; $20^{\circ}\text{--}30^{\circ}$ : 5; $30^{\circ}\text{--}40^{\circ}$ : 7; $40^{\circ}\text{--}50^{\circ}$ : 11; $50^{\circ}\text{--}65^{\circ}$ : 8) |
| Maximum tilt angle ( $^{\circ}$ ) | 65                                                                                                                                                                                                                                 |
| Resolution limit for merging (Å)  | 6.0                                                                                                                                                                                                                                |
| No. of reflections merged         | 33,298                                                                                                                                                                                                                             |
| No. of independent phases         | 3,848 (up to $8\text{ Å}$ )                                                                                                                                                                                                        |
| Overall weighted phase residual   | 29.0% (up to $8\text{ Å}$ )                                                                                                                                                                                                        |
| in resolution range (Å):          |                                                                                                                                                                                                                                    |
| 200.0–14.0                        | 25.3%                                                                                                                                                                                                                              |
| 14.0–10.0                         | 32.6%                                                                                                                                                                                                                              |
| 10.0–8.2                          | 36.7%                                                                                                                                                                                                                              |
| 8.2–7.1                           | 36.7%                                                                                                                                                                                                                              |

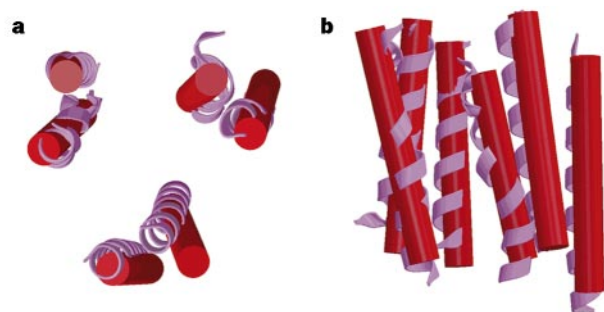


**Figure 2** Comparison of D1–D2 transmembrane helices with the purple bacterial reaction centre. **a**, View from the luminal side. Cylinders represent the D1 (yellow) and D2 (orange) helices, as in Fig. 1. Membrane-spanning segments of the L (dark blue) and M (light blue) subunits of *R. viridis* reaction centre are drawn as ribbons. The numbers indicate the order of helices in L and M. **b**, Side view.

A comparison of the D1–D2 helices with the L and M subunits of the purple bacterial reaction centre (Fig. 2) reveals the striking similarity between the two complexes<sup>9</sup>. Differences in the lateral positions of helices are no more than 3.5 Å and are mainly restricted to helices 1 and 4 of the D2 subunit and to helix 4 of the D1. The redox-active tyrosines that mediate electron transfer to cytochrome P680 of PSII are thought to be located near the luminal ends of helix 3 of the D1 and D2 proteins<sup>1</sup> and there seem to be structural differences between the PSII and bacterial proteins in this region. In the case of the D1 protein, this location is the binding site for the manganese cluster<sup>10</sup>.

A recent X-ray map of the photosystem I (PSI) reaction centre at 4 Å resolution<sup>11,12</sup> revealed an unexpected structural homology of five out of the eleven membrane-spanning helices of the PsaA and PsaB proteins to the purple bacterial reaction centre L and M subunits, showing the same local near two-fold symmetry. These portions of the PsaA and PsaB proteins are therefore also similar to the D1 and D2 proteins in our PSII map. However, although the position and orientation of the helices near the local two-fold axis are quite similar in PSI and PSII, the outer PSI helices are more tightly grouped than the outer PSII helices, resulting in an inward radial displacement by up to 8.5 Å. As a result, this central set of 2 × 5 helices appears to be considerably more compact in PSI. The similarity between the purple bacterial reaction centre and PSII is thus more pronounced than that between PSI and the bacterial reaction centre.

A comparison of the six helices ascribed to CP47 in our map and the first six helices of the PSI reaction centre proteins (Fig. 3a, b) shows a surprising structural similarity. The fit is excellent, except that one of the CP47 helices seems to be tilted slightly more towards the outside of the complex on the luminal side than does its PSI counterpart (Fig. 3a). The first six helices of the *psaA* and *psaB* gene products share weak amino-acid sequence homology with the



**Figure 3** Comparison of CP47 with the PSI inner core antenna. **a**, View from the luminal side. Red cylinders represent the six transmembrane helices of CP47. The corresponding six N-terminal helices of the PsaA protein of PSI are drawn as pink ribbons. **b**, Side view.

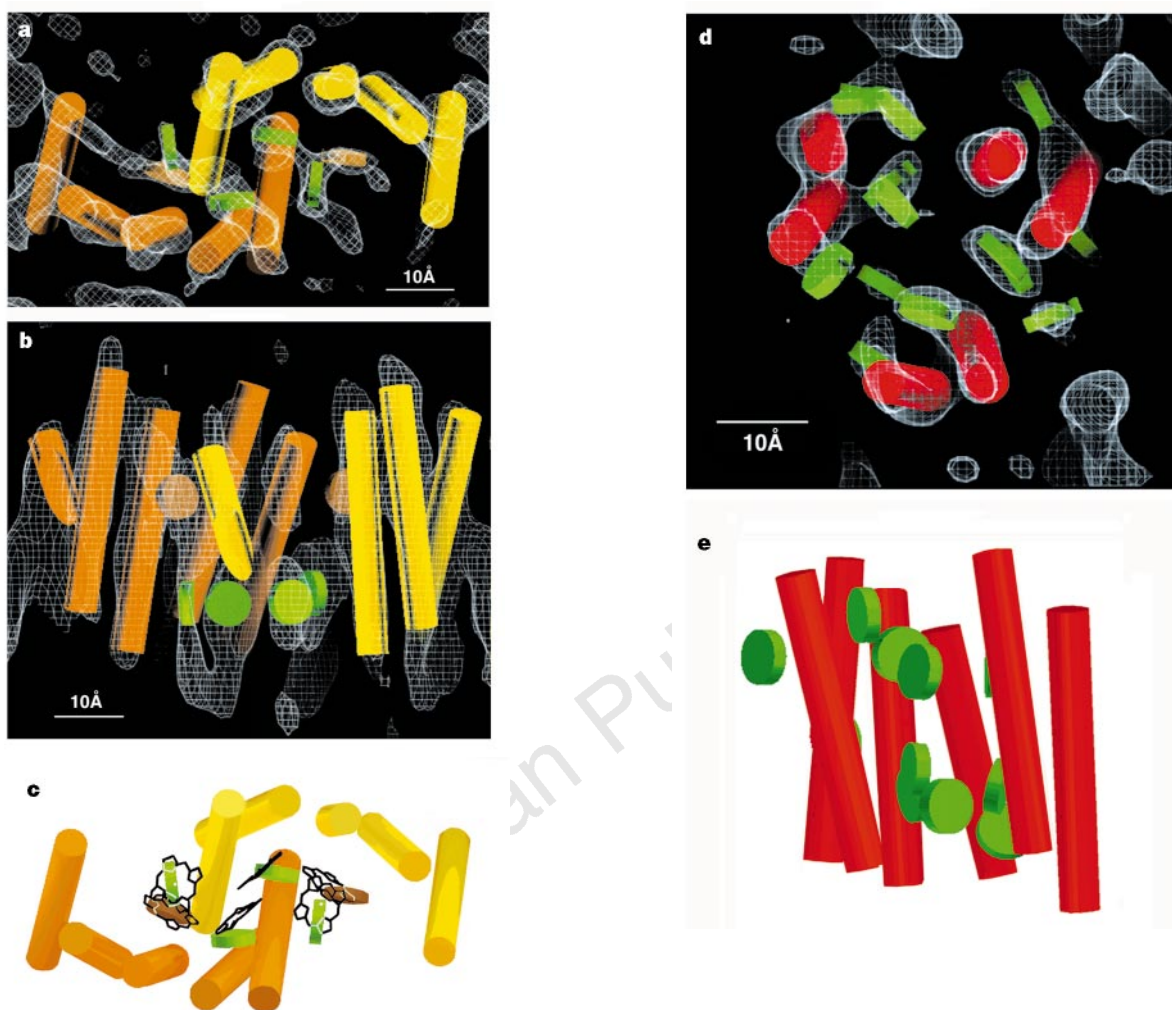
predicted helices of the core antenna proteins, CP47 and CP43, of PSII (refs 13, 14). This observation, and the similar arrangement of these helices in the two complexes, confirm our assignment of these six helices to CP47. As shown in the projection map<sup>15</sup>, this group of six helices is related to the corresponding PSI structure by a 15° in-plane rotation. On the basis of crosslinking experiments<sup>16</sup> and other studies<sup>17</sup>, it is thought that CP47 is closer to the D2 protein than to other proteins in the PSII complex. We conclude that the orange subunit adjacent to CP47 is likely to be D2. D1 would then correspond to the yellow subunit.

Although the six helices of CP47 show clear structural similarity to the amino-terminal helices of the PSI reaction centre proteins, CP47 has a large hydrophilic loop of nearly 200 residues, linking helices 5 and 6 (ref. 6). Because of the anisotropy of resolution, we expected loop densities to be less visible than membrane spans in our three-dimensional map. In the side view (Fig. 1a), however, two of the rods assigned to CP47 are significantly longer than the other transmembrane helices and continue on into the thylakoid lumen. It is therefore likely that these two densities belong to helices 5 and 6 of CP47. These two helices face the D1–D2 heterodimer, indicating that the large luminal loop joining helices 5 and 6 would be adjacent to the reaction centre. The stromally exposed C terminus of CP47 would be close to the N terminus of the D2 protein, as might be expected by analogy with the structure of the PSI reaction centre proteins<sup>14</sup>.

The similarity between the CP47 helices and the corresponding helices in PSI supports the idea that, in a complex containing both core antenna complexes, the putative six transmembrane helices of CP43 lie on the other side of the D1–D2 heterodimer to CP47. CP47 and CP43 would then share the same near two-fold symmetry that relate the D1 and D2 proteins to one another<sup>14,15,18</sup>, resulting in a corresponding symmetry in the transfer of light energy from these inner antenna proteins to each side of the PSII reaction centre.

The space within the ten helices of the D1–D2 protein contains several smaller regions of density of a roughly oblate ellipsoid shape. These regions probably represent the head groups of the non-covalently bound tetrapyrrole pigments. The 6 Å map of light-harvesting complex II (LHCII) (ref. 4) indicated that chlorophyll tetrapyrroles can be seen clearly in an electron crystallographic map at this resolution. They would also be expected to be visible, albeit less clearly, in the present map at an in-plane resolution of 8 Å.

A comparison of the positions of the six tetrapyrrole pigments in the purple bacterial reaction centre with the densities in the D1 and D2 subunits indicates, in each case, the presence of a corresponding tetrapyrrole in PSII (Fig. 4a–c). Although the inherent noise level partially obscures some of the associated features, we are confident that the densities in PSII nearest to the position analogous to that of the bacterial special pair belong to two chlorophylls. These two chlorophylls are near the luminal membrane surface, where they



**Figure 4** Pigments in the CP47 reaction centre complex. **a**, View of the D1-D2 map region from the luminal side, with fitted transmembrane helices (yellow and orange), chlorophylls (green discs, diameter 6.6 Å) and pheophytins (brown discs). **b**, Side view of the D1-D2 map region. **c**, View as in **a**, but without

the map and showing purple bacterial reaction centre cofactors in black. **d**, View from the luminal side of the CP47 map region, with transmembrane helices (red) and chlorophylls (green). **e**, Side view of CP47 helices and chlorophylls.

would be in close proximity to the water-splitting manganese cluster. The centre-to-centre distance between them appears to be  $\sim 11$  Å, which is large than the distance of 7.6 Å between the members of the bacterial special pair. The orientation of the two chlorophylls is unknown at present. The radical cation designated  $P680^{+}$  is probably located on the chlorophyll close to Tyr 161 of the D1 protein, as the side chain of Tyr 161 acts as the electron donor<sup>1,19</sup>. Regarding the chlorophylls that are structurally and, presumably, functionally homologous to the accessory bacteriochlorophylls in the purple bacterial reaction centre, there is good density for one of the PSII chlorophylls and weaker density for the other (Fig. 4a, b). The overall arrangement of the chlorophylls in the D1-D2 heterodimer, and in particular the distance between the central pair, is consistent with the weak exciton coupling of  $P680$  that distinguishes this oxygenic reaction centre from its bacterial counterpart<sup>1,20</sup>.

Two apparent pigment densities near the centre of the membrane are attributed to pheophytins (coloured brown in Fig. 4b), which are known to be present in the D1-D2 heterodimer<sup>21</sup>. Their position corresponds to that of the bacteriopheophytins in the *Rhodospseudomonas viridis* reaction centre (Fig. 4c). Spectroscopic data indicate that one of the pheophytins in PSII acts as the primary electron acceptor, just as bacteriopheophytin does in purple bacterial. As in the case of the chlorophylls, the exact orientation of the pheophytins cannot be deduced at this resolution. The space within the six helices of CP47 (Fig. 4d, e) also contains several similar

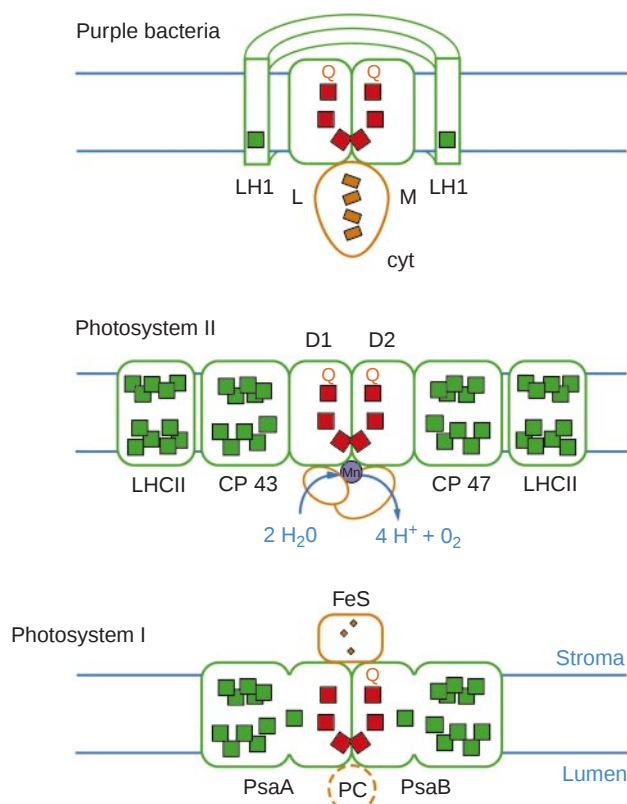
densities, 14 of which are tentatively assigned to chlorophylls, in agreement with biochemical data suggesting a similar number<sup>7</sup>.

Our results demonstrate a close evolutionary link between the three types of reaction centre in photosynthetic organisms: there is a link between PSII and the purple bacterial reaction centre on the one hand, and between PSII and PSI on the other (Fig. 5). A direct evolutionary relationship between PSII and the purple bacterial reaction centre has long been postulated<sup>5,18</sup>, but, in the absence of structural data, the extent of the similarity remained unknown. Our map indicates a high degree of structural similarity between the purple bacterial reaction centre and PSII. This means that the five-helix scaffold of the two reaction centres may have been largely unchanged since photosynthesis first evolved 3.5 billion years ago. The similarity between CP47 and the corresponding six helices of the PsaA-PsaB proteins suggests that the latter arose by genetic fusion of a CP43/CP47-like protein with a five-helix reaction-centre prototype. Phylogenetic relationships between the photosynthetic bacteria<sup>22,23</sup> indicate that the *Chloroflexaceae*, which have a reaction centre similar to that of purple bacteria, are the most ancient photosynthetic organisms on Earth, consistent with the idea that PSI evolved from a PSII-type reaction centre. □

#### Methods

**Specimen preparation and electron cryomicroscopy.** Two-dimensional crystals were prepared for electron cryomicroscopy as described<sup>15</sup>, except that





**Figure 5** Comparison of the three types of reaction centre found in photosynthetic organisms. Top, bacterial reaction centre. The L and M subunits, which bind the pigments active in charge separation (red), are related by local near two-fold symmetry. The reaction centre is surrounded by a ring of light-harvesting proteins (LH1). Electrons are fed into the reaction centre by a haem-binding cytochrome (cyt). Centre, photosystem II. The D1 and D2 proteins are structurally and functionally homologous to the L and M subunits of the bacterial reaction centre and hold the active pigments in a similar configuration. Light energy is collected by LHCI and channelled into the reaction centre by the core antenna proteins, CP43 and CP47, which are positioned at either side of the D1–D2 heterodimer. The resulting charge separation enables the manganese cluster on the luminal surface to withdraw electrons from water, releasing oxygen into the atmosphere. Bottom, photosystem I. The PsaA and PsaB proteins form a PSII-like heterodimer. PsaA and PsaB each consist of a reaction centre system equivalent to D1 or D2, and a core antenna equivalent to CP43 or CP47. Electrons are taken from reversibly bound plastocyanin (PC) on the luminal side, and delivered to the iron-sulphur clusters (FeS) on the stromal side, where they are used to reduce NADP<sup>+</sup>.

the carbon film was supported on washed 300-mesh titanium grids or 400-mesh nickel grids (Plano). Low-dose electron micrographs of crystals cooled to  $-182^{\circ}\text{C}$  were recorded<sup>15</sup> with a Philips CM200 FEG electron microscope operating at 200 kV, at a magnification of  $\times 50,000$  and with 1-s exposure time, on Kodak SO-163 film. The electron dose was  $5\text{--}10\text{ e}^{-}\text{\AA}^{-2}$ . Images at tilt angles  $>35^{\circ}$  were recorded after withdrawing the twin-blade anticontaminator. **Image processing and data analysis.** Well-ordered two-dimensional crystal areas were digitized with a Zeiss SCAI scanner with a linear CCD array at a size of  $7\text{ }\mu\text{m}$  per pixel, corresponding to  $1.4\text{ }\text{\AA}$  on the specimen, and processed using MRC programs<sup>24</sup>. We selected 45 images in total with tilt angles from  $0^{\circ}$  to  $65^{\circ}$ , taking into consideration both the angular distribution of the tilt-axis angles and the tilt angles to fill up Fourier space effectively (Table 1). Image data from another batch of two-dimensional crystals used in the earlier projection analysis<sup>15</sup> were not included because the unit cell dimension in one direction differed by  $\sim 8\%$ . Tilt angles  $>20^{\circ}$  were initially calculated from the lattice parameters<sup>25</sup>. Tilt angles  $<20^{\circ}$  were estimated from the defocus in the four corners of the negative. Processed image areas were corrected for image distortions, specimen tilt and objective lens astigmatism as described in refs 26,

27. We obtained 7,094 independent phases (from 33,298 measurements) from 45 processed images, including all data with IQ values between 1 and 8, with an overall phase residual of  $29.4^{\circ}$  (Table 1). Curves representing the variation of phases and amplitudes along lattice lines were fitted to data points using the program LATLINE<sup>28</sup> and sampled at intervals of  $0.005\text{ }\text{\AA}^{-1}$ . Structure-factor amplitudes were scaled using a temperature factor of  $B = -500\text{ }\text{\AA}^2$  to compensate for resolution-dependent fade-out of image intensities. Data between  $6\text{ }\text{\AA}$  and  $8\text{ }\text{\AA}$  resolution were weak and increased the noise level of the three-dimensional map while not revealing significant new map features. Data were therefore truncated to  $8\text{ }\text{\AA}$  resolution. The point-spread function of this data set shows that the effective resolution perpendicular to the membrane plane is  $13.6\text{ }\text{\AA}$ . We calculated a Fourier three-dimensional density map from 3,576 phases and amplitudes using standard CCP4 routines.  $\alpha$ -Helices of 24 residues were fitted to map features using the graphic program O (ref. 29).

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# Immunology answers

New products that use antibodies and help to study of the immune system include a consolidated staining system, an integrated microplate imaging system and software for microscopic image analysis of enzyme-linked immunospots.

## Emerging Models Program

From Taconic [www.taconic.co.uk](http://www.taconic.co.uk)

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This programme has been designed to assist investigators in establishing a clearly defined health and genetic profile for their new transgenic models, and then to facilitate the sharing of these models between laboratories. Taconic assists all participants with intellectual property considerations, which often pose challenges to research programmes using transgenic animals, and makes previously unavailable models accessible. Ron Schwartz, head of the National Institute for Allergy and Infectious Diseases' (NIAID) Laboratory of Cellular and Molecular Immunology states: "we saw that a commercial breeder could help resolve some of the supply-demand problems and this programme makes new models available for purchase at low cost". Several immunology models that were originally developed by NIAID for internal use are now maintained at Taconic as part of the NIAID Genetic Repository. The Taconic Emerging Models Program establishes a partnership between investigators and Taconic: owners share their transgenic models with other researchers, yet retain control of the distribution terms, production levels and price of each model. Taconic also handles intellectual property considerations so that an emerging model can be distributed without infringing patent rights that may apply.

**Reader Enquiry No. 100**

## Immuno instrumentation

### OptiMax Plus

From BioGenex [www.biogenex.com](http://www.biogenex.com)

*A consolidated staining system with new features important to research and clinical laboratories*

BioGenex claims that this is the only system with an on-board dewaxing capability, providing an automated processing step for removal of paraffin from the tissue slides. The OptiMax Plus system incorporates slide dewaxing, IHC, special stains, H&E (haematoxylin and eosin) staining and *in situ* hybridization in a single compact instrument. The stainer is available in two system configurations: an optimized bar-code system and an 'open' system. The bar-code system is well suited to routine applications with labels on reagent vials providing product identification, volume



Maximize staining with OptiMax from Biogenex.

and expiration date; the bar-code labels on slides identify the antibody protocol. The open system configuration includes a programmable user interface for random access operation, which allows for more complex and customized protocols. The OptiMax Plus consolidated staining system is intended for *in vitro* diagnostic use, and uses removable slide and reagent racks. Printouts, including case number and assay data, provide quality-control and quality-assurance documentation consistent with the current CAP regulations. The company also offers automated stains kits, which are intended to replace complex, labour-intensive manual staining methods. Kits that are currently available include acid fast, amyloid (Congo Red), mucicarmine, Gomori's trichrome, periodic acid Schiff (with or without digestion), Masson's trichrome, iron stain and Alcian blue. These stains kits are for *in vitro* diagnostic use, and only for use on the OptiMax Plus consolidated staining system.

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### Ultramark microplate reader

From BioRad [www.bio-rad.com](http://www.bio-rad.com)

*A microplate imaging system that can read any plate format up to 3,456 wells, with a stated linear absorbance up to 4.5*

Designed to help increase speed and flexibility in high-throughput screening techniques, this new microplate reader can operate as an imager, as well as carrying out normal-point readings, thus providing capabilities for cell screening, colony picking and colorimetric assays. The system has stated scan times of 6 s for a single-wavelength 96-well plate and 13 s for dual-wavelength plates — drug candidate screening can be increased further by linking the Ultramark to a robotics system, says Bio-Rad. Running on Microplate Manager software, simultaneous data analysis can be conducted on as many as 12 separate assays from a single plate.

Other analysis functions include a choice of curve-fit programs and complex kinetic analysis with real-time data acquisition and zoom-to-a-well capability.

**Reader Enquiry No. 102**

### Autostainer XL

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lation and cancer treatment follow-up are available, as are full Westguard quality-control options. PC Anywhere software enables remote system diagnostics to be performed, allowing technical specialists from anywhere in the world to link with the system and perform diagnostic routines.

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## KS-Elispot

From Carl Zeiss Vision [www.zeiss.de](http://www.zeiss.de)

*New software for routine laboratory microscope image analysis of Elispot*

Elispot (enzyme-linked immunospot) version 4.0 allows image acquisition, spot recognition and the differentiation of incorrectly identified objects. It displays results, provides hardcopy output and enables data transmission. The easy-to-use software runs on Windows95 or NT and the software requires only the information needed for the examination of a specimen for a complete setup. The evaluation is conducted automatically on an Axioplan 2 automated, universal microscope, which must be equipped with an incident light system, a motorized stage and an autofocus device. The Elispot assay was developed to detect and quantify individual antibody-producing T or B cells. According to the manufacturer, modifications of the original test can increase the sensitivity to the extent that even cells producing minute amounts of a specific protein can be detected. This technique is stated to increase sensitivity by up to 200 times. Moreover, it can be used to detect and quantify individual T lymphocytes, which generate cytokine spots *in vitro* as a result of antigen contact. Microtitre plates with nitrocellulose membrane substrates are measured in the microscope, the immunochemically stained cytokine spots are filmed by a three-chip, CCD colour camera, digitized and evaluated by the KS-Elispot program. The spots can be measured reproducibly down to a minimum diameter of 30 µm. The individual well is scanned at a high magnification, and the individual images are then combined into an

overall image, which is then evaluated. (The various magnification steps are visible on the monitor.)

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## Assays and antibodies

### Immunoassays and antibodies

From Serotec [www.serotec.co.uk](http://www.serotec.co.uk)

*Immunoassays and monoclonal antibodies for research into the human reproductive system*

Products in this area include the inhibin A ELISA, inhibin B ELISA, activin A ELISA, pro-α C ELISA, inhibin βA subunit monoclonal antibody, inhibin βB subunit monoclonal antibody and inhibin α subunit monoclonal antibody. (These products are distributed on an exclusive basis for Oxford Bio-Innovation.) The ELISAs (and antibodies) should prove useful in investiga-



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tions of menstrual cycle, testicular and ovarian damage during chemotherapy, prenatal screening for Down's syndrome and prenatal screening for pre-eclampsia. Moreover, they should find use as markers for Sertoli cell function in men, predicting the outcome in GnRH replacement therapy for hypogonadism and predicting the outcome of *in vitro* fertilization.

**Reader Enquiry No. 107**

## Emmprin monoclonal antibodies

From Chemicon [www.chemicon.com](http://www.chemicon.com)

*Matrix metalloproteinases (MMPs) play an important role in cancer invasion and metastasis*

These enzymes may be released directly from tumour cells, or more often from fibroblasts that have been stimulated with extracellular matrix metalloproteinase inducer produced by cancer cells. Emmprin has been identified in human lung, breast, bladder and skin cancer biopsy tissues. According to the manufacturer, *in vitro* studies have demonstrated that Emmprin stimulates human fibroblast production of interstitial collagenase, stromelysin-1 and gelatinase A, which are essential for tissue degradation and remodelling during tumour invasion and wound healing. In malignant neoplasms, Emmprin stimulates MMP production by peritumoural fibroblasts, thus enhancing cancer invasion and metastasis, says Chemicon. It has also been proposed that Emmprin plays a role in tumour angiogenesis by inducing the degra-

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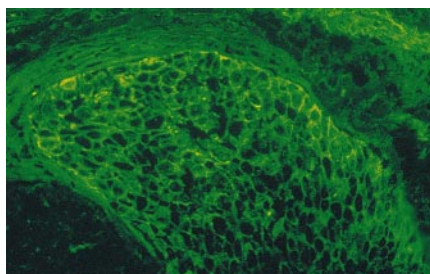
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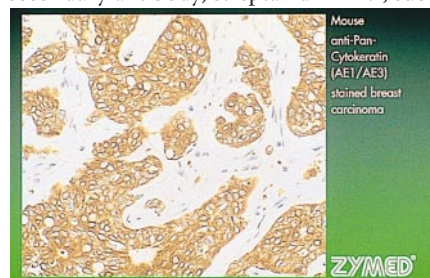
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Hats off for Zymed's offers Cap-Plus kits.

strate buffer, DAB concentrate and hydrogen peroxide. The buffer kit includes buffers 1, 2 and 3, water wash, hydrogen peroxide and haematoxylin. According to Zymed, these kits show sensitivity equal to or greater than existing reagent systems, with virtually no background. Cap-Plus kits can also be used on other vertical capillary action automated immunostainers.

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Reader Enquiry No. 113

## Immunoassay catalogue

From R&D Systems [www.rndsystems.com](http://www.rndsystems.com)

The 1998/99 edition includes two recently launched immunoassays

The new Quantikine Ab assay gives semi-quantitative detection of human auto-antibodies, whereas the QuantiGlo chemiluminescent immunoassay is formulated for human cytokines and related factors. The catalogue offers information on analyte-specific immunoassays for human, mouse and rat cytokines, as well as for cytokine receptors, adhesion molecules, MMPs, eicosanoids, cyclic nucleotides, free radicals nitric oxide and chemiluminescent ELISAs. R&D Systems' catalogue also covers human cytokines, autoantibodies to human cytokines and *in vitro* diagnostic ELISAs.

Reader Enquiry No. 114

## Rat interferon- $\gamma$ DuoSeT

From Genzyme [www.genzyme.com](http://www.genzyme.com)

A new ELISA development system for rat interferon- $\gamma$  (IFN- $\gamma$ )

This system contains the basic components required to develop sandwich ELISAs that measure natural and recombinant rat IFN- $\gamma$  in cell culture supernatants, plasma (citrate) and serum. It comes complete with a capture antibody, a detection antibody, HRP-streptavidin detection reagent and an amino-acid-analysed, mass-calibrated standard. The matched, pretested reagents are validated through Genzyme's 'proof-of-performance' testing series. According to



Multiple Duos: Genzyme's DuoSeT ELISA development systems for rat, mouse and human.

the manufacturer, the reagents provide consistent results, save development time and reduce the variation often seen with uncalibrated standards or with standards calibrated to bioassay methods. Antibody concentration may be altered as desired to affect curve height and the dynamic range of the assay. Conditions may also be varied to optimize assay performance — sufficient reagents are supplied to generate at least fifteen 96-well immunoassays when performed according to the provided protocol. Other Duo SeT ELISAs include those for mouse interleukin-10, human TGF- $\beta$ 1 and human interleukin-5.

Reader Enquiry No. 115

## LeucoCount assay

From Becton Dickinson (BDIS) [www.bd.com](http://www.bd.com)

In vitro diagnostic flow cytometric method for counting residual white blood cells (rWBCs) in platelet and red cell packs for transfusion

The LeucoCount assay eliminates the need for a separate counting standard by combining the absolute counting technology of TruCount tubes with the LeucoCount reagent. The assay has been optimized to stain residual white blood cells in leuco-reduced blood products. According to the manufacturer, the assay provides excellent precision, linearity and accuracy at clinically

relevant levels of detection. A single reagent and protocol are utilized for both platelet and red blood cell samples, reducing risk of errors in sample preparation.

Reader Enquiry No. 116

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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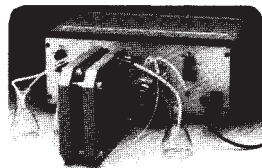
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